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C. F. A. PANTIN, Sc.D., F.R.S.

JOHN R. BAKER, D.Sc., F.R.S.

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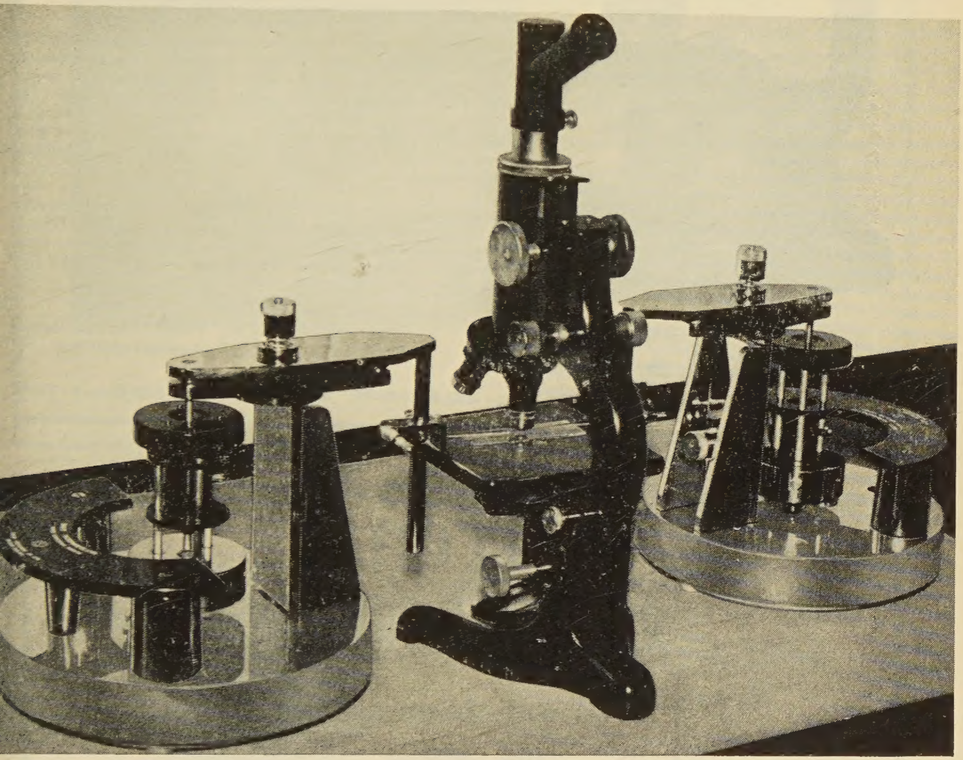

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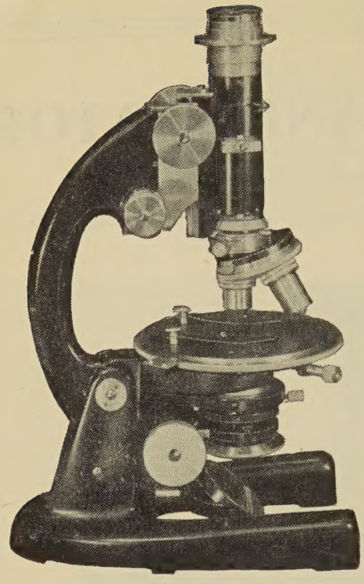

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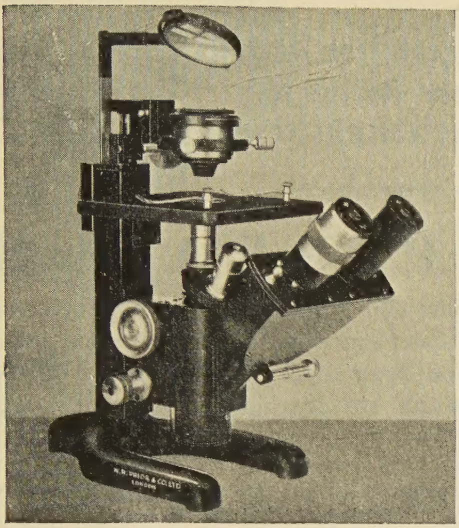

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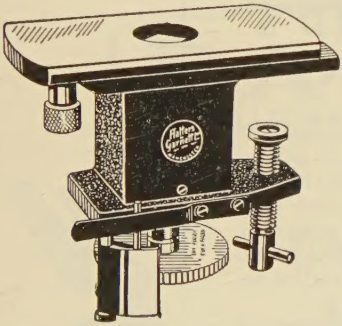

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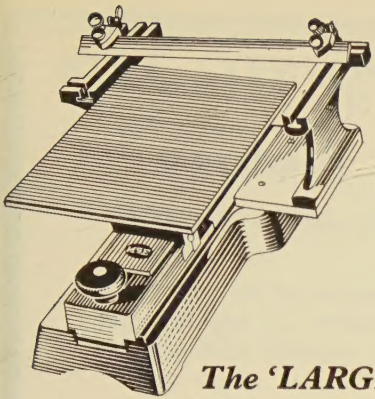

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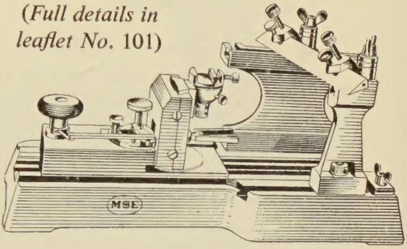

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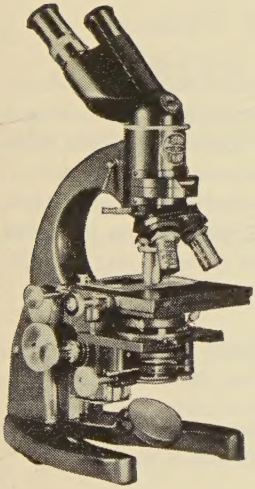

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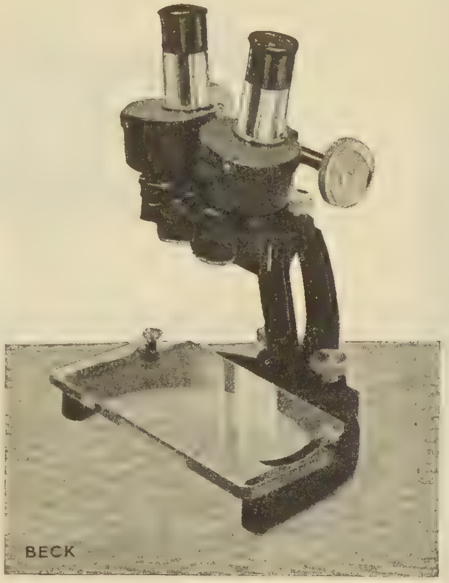

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A Cytological and Histochemical Study of the Connective-Tissue Fibres of the Leech, *Hirudo medicinalis*

By S. BRADBURY

(*From the Cytological Laboratory, Department of Zoology, University Museum, Oxford*)

With two plates (figs. 1 and 4)

SUMMARY

The fibres and the connective-tissue ground-substance of *Hirudo medicinalis* resemble those of the rhynchobdellid leech *Glossiphonia complanata* in general form and chemical composition. The fibres show a differentiation into 'cortex' and 'medulla'. The 'cortex' is found to contain arginine, and acid mucopolysaccharide but no tyrosine or lipids; these results suggest that it is collagenous. Confirmation of this supposition is provided by the X-ray diffraction pattern. The 'medulla' is an extension of the cell-body of the fibrocyte.

The cytoplasm of the fibrocyte is found to contain three types of inclusion: mitochondria; spherical lipochondria about 1 μ in diameter, which seem to consist of phospholipid; and larger triglyceride droplets. The cytoplasm also contains diffuse phospholipid and granular accumulations of acid mucopolysaccharide.

The connective tissue ground-substance resembles that of *Glossiphonia* in its properties and chemical composition, though there seems to be a higher proportion of acid mucopolysaccharide in the ground-substance of *Hirudo*.

THE presence of fibres in the connective tissue of leeches has been noted by many authors since Ray Lankester (1880) first figured them in his paper on the histology of *Hirudo medicinalis*. Bourne (1884) and Scriban and Autrum (1934) in particular deal with their general structure, but as far as is known, no detailed histochemical study has been carried out on the fibres of this species of leech. Recently an account was published (Bradbury, 1957) describing the histochemistry of similar fibres which occur in the rhynchobdellid leech, *Glossiphonia complanata*, together with details of the ground-ubstance in which they are embedded. It is the purpose of this paper to give a comparable account of the fibres, fibrocytes, and intercellular matrix in the gnathobdellid leech *H. medicinalis*.

MATERIAL AND METHODS

Though the medicinal leech no longer occurs abundantly in this country, pecimens may readily be obtained from dealers and kept alive in aquarium ars in the laboratory for many months. This species is much larger than the pond leech *G. complanata*, and it has the advantage that the connective tissue may be dissected out so that the living cells may be studied. In the present work, small pieces of connective tissue were removed and placed in 0·75% aline solution so that any pieces of adherent muscle or gut-wall could be emoved before the connective tissue was mounted in the saline. Light pressure

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on the coverslip was sufficient to spread the tissue thinly enough for satisfactory phase-contrast microscopy. Interference and dark-ground microscopy were also used in the study of the fibrocytes. For supravital staining, neutral red chloride, methylene blue, dahlia violet, and Janus green B were used. The dyes were dissolved in distilled water, which was then diluted with saline to give the required concentration, according to the technique adopted in our laboratory (Baker, 1949; Chou, 1957). It was found that a period of 20 min. in the dye gave satisfactory results, though in the case of Janus green the tissue often had to be left as long as one hour in order to achieve any staining.

Details of the histochemical tests applied to fixed material are listed in the appendix.

THE CONNECTIVE-TISSUE FIBRES

The connective-tissue fibres may be seen in any transverse section coloured by suitable techniques, or in spreads of connective tissue studied by phase-contrast or interference microscopy. The fibres are single and interlace extensively in the connective-tissue ground-substance which fills the bulk of the body. In all essential details, the fibres of *Hirudo* appear identical with those found in the other species of leech. They are very long, about 1–2 μ in diameter, and show the 'cortex' and 'medulla' appearance noted before (Bradbury, 1957). There is no branching, and no tendency for the fibres to run together (fig. 1, A), as in the tendons of vertebrates. From a careful study of sections it seems that the tendency noticed in *Glossiphonia* for the fibres to be orientated obliquely to the long axis of the body, and to show 'crossing over' with fibres on a different plane, is not very marked in the medicinal leech. It seems that the fibre distribution is much more random, which may, perhaps, be partly responsible for the very marked difference in 'body tone' which is found in these two species; for *Hirudo* always appears much more limp than *Glossiphonia*. It was not possible to observe the mode of termination of the fibres.

The staining reactions of the fibres were those typical of collagen, though it was noticed that there was no birefringence of the fibres when they were studied with polarized light; they did swell, however, on treatment with acetic acid. Such reactions only provide presumptive evidence of the nature of the fibre, so further study was by means of more specific tests.

Histochemistry of the fibre cortex

As shown in the appendix, there was no positive reaction to any of the tests for lipids. It was suspected that some lipid material could be present in the fibre in such a condition that any reaction with the standard colouring agents for lipids would be prevented; the sections were therefore treated by the

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FIG. 1 (plate). A, fibres in the connective tissue of *Hirudo*, photographed with the interference microscope. Note the cortex and the random arrangement of the fibres.

B, a fibrocyte, showing the numerous triglyceride droplets in the cytoplasm. This was a living preparation photographed with the interference microscope.

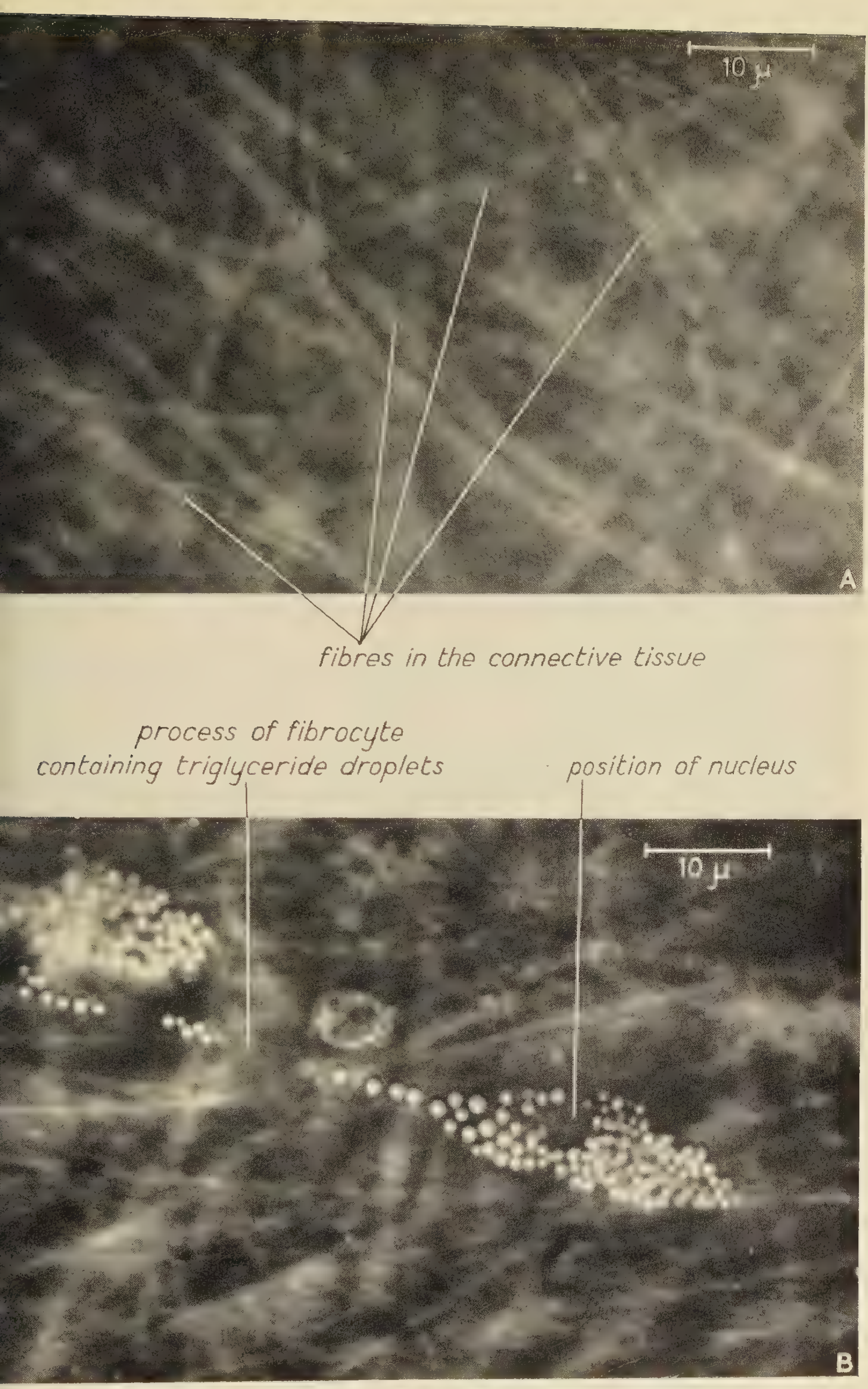

10 μ
A
fibres in the connective tissue
process of fibrocyte containing triglyceride droplets
position of nucleus
10 μ
B

FIG. 1

S. BRADBURY

echniques of Ciaccio (1926) in order to unmask any lipids. After fixation in Da Fano's fluid or in Cajal's fluid it was not possible to demonstrate any lipid eaction in the fibre 'cortex'. As suggested by Ciaccio, tissues were fixed in ormaldehyde and sections were then incubated in a solution of phenol (1% in distilled water) for 24 h, a procedure which he considered to break most associations of lipid and protein. As there was still no positive reaction from the cortex of the fibres, it was concluded that they do not contain any appreciable amount of lipid.

Proteins and amino-acids

As in the previous study of the fibres of *Glossiphonia* only three methods were tried, namely the Sakaguchi test for arginine (Baker, 1947), the Hg / nitrite test for phenols, especially tyrosine (Baker, 1956), and the coupled tetrazonium reaction (Danielli, 1947; Pearse, 1954). Positive results were obtained from the Sakaguchi test, but no reaction could be obtained with the Hg / nitrite test. There was a positive reaction with the coupled tetrazonium technique, which was slightly increased by the application of mild heat and benzoylation. This is considered to be suggestive of the presence of collagen or some related protein (Pearse, 1954). It is also of interest to note that these histochemical tests suggest that the quantity of arginine is much greater than that of tyrosine, a further point which lends support to the hypothesis that collagen is present in the cortex of these fibres.

In an attempt to measure the basiphilia of the cortex, the methylene blue extinction technique of Pischinger (1926) and of Dempsey, Singer, and Wislocki (1950) was used. This method can be adapted to furnish an approximate indication of the iso-electric point of a tissue component. In the present material, it was found that the cortical material of the fibres ceased to take up the dye at pH 5, which is remarkably close to the value for the iso-electric point of collagen (pH 4·85) as given by Pearse (1954).

Carbohydrates

The fibre cortex gave a positive reaction to the periodic acid / Schiff (PAS) test, appearing slightly more coloured than the ground-substance, which also gave some reaction. The PAS reaction was also positive after the slides had been incubated in saliva at 37° C, so that the result was not due to the presence of glycogen. As no lipid material could be detected, it seems unlikely that the reaction was given by glycolipids, but was probably caused by the presence of mucopolysaccharide in or on the fibres.

When the sections were stained with toluidine blue, it was found that there was a slight metachromasy of the cortex, which could be abolished by a treatment with hyaluronidase (a solution of 'Rondase' 1 mg / ml) for 1 h at 37° C. This suggests that some part at least of the PAS reaction is due to the presence of an acid mucopolysaccharide. As the specificity of this enzyme has not been established with certainty, it is not possible to be more precise, though

Pearse (1954) considers it probable that such a substance would be either hyaluronic acid itself, or chondroitin sulphate of type A or C.

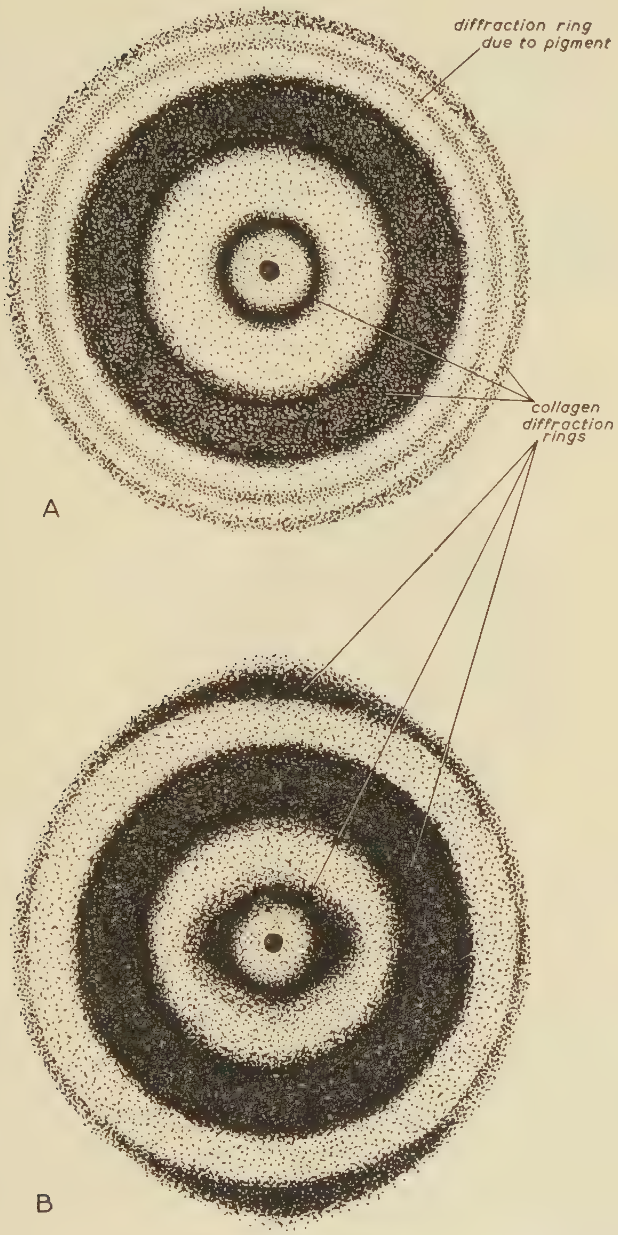
Miscellaneous tests

Although it was not thought likely that there would be any striking accumulation of nucleic acids in the cortex of the fibre, the Feulgen and pyronin / methyl green tests were carried out. With the Feulgen test for deoxyribonucleic acid (DNA) there was a positive result in the nuclei of the fibrocytes, but it was noted that this reaction was only feebly positive, as in most of the other somatic nuclei. When the pyronin / methyl green technique of Jordan and Baker (1955) was employed, it was found that both the ground substance, the cortex of the fibres, and the cytoplasm of the fibrocytes coloured with the pyronin. In order to discover whether this reaction was, in fact, due to the presence of ribonucleic acid (RNA), the 'treated-saliva' technique was used (Bradbury, 1956). It has been shown that the basiphilia due to RNA can be abolished by incubating slides in a bath of treated saliva; in the cortical material of the fibres and in the cells it was found that the basiphilia resisted the incubation, and was not diminished, so there does not seem to be any RNA present in this material. It seems likely that the strong affinity for pyronin may be attributed to the presence of large amounts of acid mucopolysaccharides, as these substances would be expected to show a strong basiphilia. It is also perhaps significant that the epidermal mucous-cells containing large quantities of strongly metachromatic material, which can be shown to be acid mucopolysaccharide, also colour strongly with pyronin.

As it proved possible to extract pieces of connective tissue, it was decided to try to obtain the X-ray diffraction pattern of the fibre. It has been shown by numerous workers (e.g. Astbury, 1938; Champetier & Fauré-Fremiet, 1938) that proteins of the collagen group have a very characteristic X-ray diffraction pattern, so that here is a further criterion for the identification of collagen, which has the advantage of being independent of a chemical reaction.

Small pieces of tissue were excised and placed in distilled water; all adherent muscle was removed. In order to remove all salts and as much of the pigmented tissue as possible, both of which would interfere with the diffraction pattern of the protein, the tissue was washed for 72 h in running water and then in distilled water (several long washes). The tissue was dried down on a glass slide and finally lifted off this surface with a sharp scalpel. The exposure to the X-ray beam lasted about 24 h.

The resulting diffraction pattern was that of a protein of the collagen type; it is shown in fig. 2 together with the diffraction pattern of human articular cartilage (chiefly collagen and ground-substance) for comparison. It is seen that the same rings are present in both, though in the leech preparation there is an extra ring due to the pigment; the degree of orientation of the collagen is only small. Both of these diffraction patterns correspond very closely to the 'supercontracted' type of collagen diffraction pattern, noted by Astbury in the reference already quoted.



IG. 2. A, the X-ray diffraction pattern of the connective tissue of *Hirudo*. Note the three llagen rings and the additional ring which is due to the presence of pigment. B, the X-ray ffraction pattern of human articular cartilage for comparison. Only the three collagen rings are present. Both figures are semi-diagrammatic.

On the basis of this evidence, together with consideration of the histochemical and other studies, it seems possible to say with certainty that the cortex of the connective-tissue fibres of *Hirudo* is a collagen-type protein, and that it is associated with some quantity of an acid mucopolysaccharide, either incorporated into its substance, or adsorbed on to its surface.

THE BODY OF THE FIBRE-CELL

General

The shape and general structure of the cell-body is shown in fig. 1, B and in a diagrammatic form in fig. 3; it is usually between 20 and 24 μ in length with a diameter of about 10 to 15 μ. The ends of the cell are drawn out into the very long processes which form the medulla of the fibres. As was noticed in the other species of leech, the cortex of the fibre is continued over the cell-body, though it is thinner in this region. The cell has one nucleus, usually oval, measuring about 5 to 8 μ along its greatest length; there is often a well-defined nucleolus.

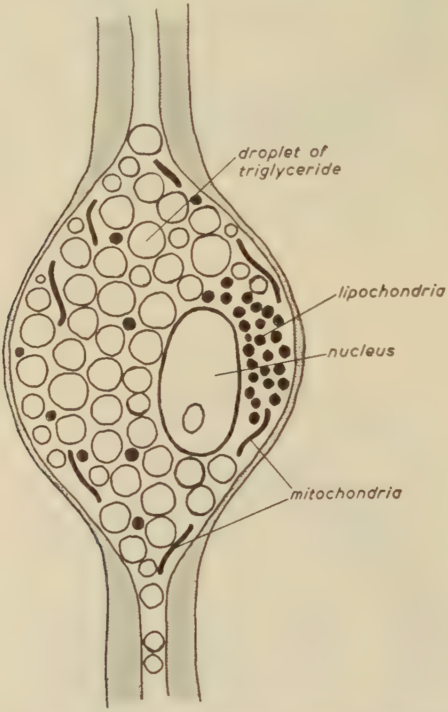


FIG. 3. A diagram of the structure and cytoplasmic inclusions of the fibrocyte of *Hirudo*.

When these cells were studied alive, by both phase-contrast and interference microscopy, it was found that three types of cytoplasmic inclusions could be distinguished. The most obvious of these are the spherical, highly refringent droplets which are very densely packed and occupy almost the

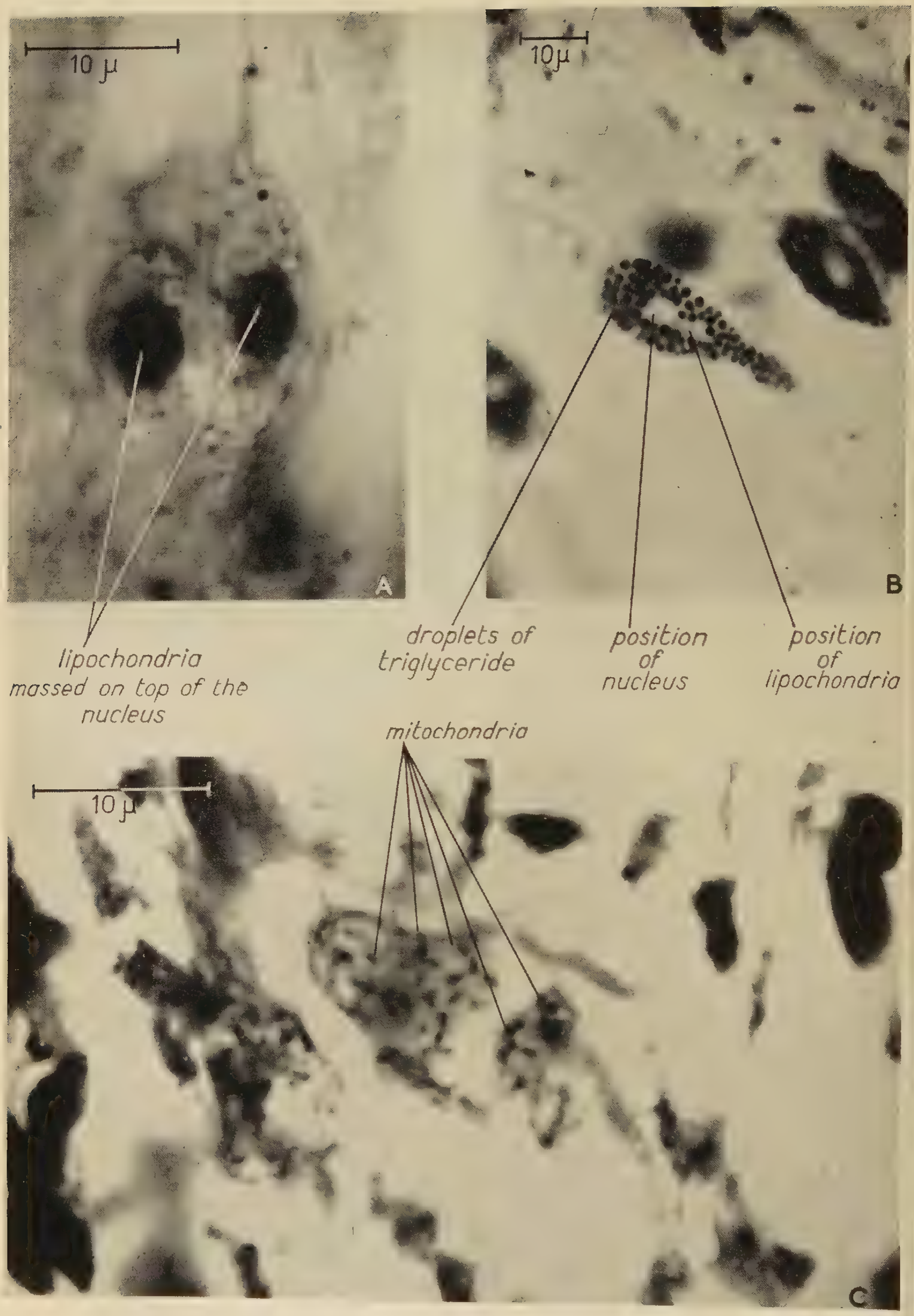


FIG. 4

S. BRADBURY

whole of the cytoplasm (figs. 1, B; 4, B). They are variable in size but their diameters seem to fall in the range 2 to 5 μ. With the interference microscope it was found possible to measure the refractive index of these droplets by the method outlined in a previous paper (Bradbury, 1957), when the mean value of several measurements gave a result of 1·486, which strongly suggests that they are lipid in nature and do not have much water associated with them (Ross and Chou, 1957).

A further type of cytoplasmic inclusion is very difficult to see on account of the great number of larger droplets, but if a preparation is well flattened either by gentle pressure on the coverslip or by allowing it to dry up slightly, then many small droplets become visible in the cytoplasm near the nucleus (figs. 3; 4, A, B). They seem to be spherical and do not exceed 1 μ in diameter; although they have a higher refractive index than the cytoplasm they are not so strongly refractive as the larger droplets in the same cell. It is noticeable that they alone colour with vital dyes such as neutral red and dahlia violet, thus contrasting sharply with the larger droplets which remain uncoloured by these techniques. It seems that these small droplets differ significantly from the other cytoplasmic inclusions, a supposition which is supported by the observation that only these small objects will impregnate with silver or osmium in the Aoyama or Mann-Kopsch techniques.

The third type of cytoplasmic inclusion is the mitochondria. They are not very numerous, but with phase-contrast they may be seen to be scattered around the periphery of the cell. They are short rods, either straight or slightly curved, measuring about 0·5 μ in diameter and about 2 μ in length. It has been possible to study these in fixed preparations by the use of the Hermann / post-osmication technique (Baker, 1957). A typical appearance is shown in fig. 4, C. The mitochondria will colour with Janus green or Janus black, though only feebly and after a long exposure to the dye.

Histochemistry

One of the chief differences from the similar type of cell in *Glossiphonia* is that in the present work it was found that the cytoplasm of the fibrocyte of *Hirudo* loses its affinity for methylene blue at a higher pH. The M.B.E. value is approximately pH 4. The cytoplasm also contains much more carbohydrate than the corresponding cytoplasm in the other species of leech. The PAS-positive substance is found to occur in the cytoplasm both in a diffuse and in a particulate form, the latter being very noticeable just below the cell surface, where small PAS-positive granules tend to aggregate. This observation is especially interesting in view of reports (e.g. by Jackson, 1955) that fibrogenesis in some avian tissues is accompanied by the secretion of droplets of

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FIG. 4 (plate). A, two fibrocytes, showing the lipid droplets massed on top of the nucleus. Acid haematein technique.

B, fibrocyte coloured with Sudan black after fixation in Da Fano's fluid. The triglyceride drops are clearly seen together with the space formerly occupied by the lipochondria.

C, mitochondria in a fibrocyte. Baker's Hermann / post-osmication technique.

PAS-positive material. In the present study the PAS-positive substance was found to be strongly metachromatic. It thus seems very probable that again the reaction may be attributed to the presence of highly sulphated acid mucopolysaccharide.

The cytoplasm also gives a strong, diffuse reaction with Sudan black. Taken in conjunction with the presence of PAS-positive substances, this is perhaps suggestive of the presence of a cerebroside. After fixation in cold acetone, however, the cytoplasm failed to take the Sudan colouring agents. It thus seems unlikely that the PAS reaction and the sudanophilia is due to the presence of a cerebroside. It was found that both types of droplet coloured with Sudan black, so that their lipid nature may be regarded as established. It was, however, difficult to study them because the diffuse lipid in the cytoplasm obscured their outline. Fixation in Da Fano's fluid followed by coloration with the Sudan dyes proved helpful in this respect. It was found that neither the cytoplasmic lipid nor the smaller lipid droplets were preserved, only the large spherical lipid drops colouring strongly (fig. 4, B). Nile blue was found to colour these drops pink. This suggests that they are neutral lipid (Cain, 1947). No reaction with this dye could be detected in any other component of the cell. There was a definite though slight coloration of the large lipid drops with the performic acid / Schiff technique and also with the PAS reaction, but it was found that in the latter test they also coloured with the Schiff's reagent when the preliminary oxidation with periodate was omitted, so that it is not possible to draw any conclusions from these observations. As the coloration of the lipid drops was most marked after fixation in formaldehyde, it may be that the formaldehyde reacted with lipid to form a PAS-positive complex, as Wolman and Greco (1952) suggested. Alternatively, the reaction may be the result of the oxidation of unsaturated lipids by atmospheric oxygen, with production of aldehydic groupings. Support for this hypothesis is provided by the observation that the reaction was much more pronounced when the sections were allowed to stand for some time before being placed in Schiff's reagent.

The large fat drops did not give any positive reaction with the acid haematein test, nor with any other test which was used in the present study, so that it seems probable that these drops are mostly triglyceride; the measured refractive index is in agreement with this hypothesis. The small lipid droplets, however, colour very strongly with the acid haematein reaction (fig. 4, A), as do the mitochondria. If this technique is used in conjunction with a lipid-soluble colouring agent such as Sudan IV, the small lipid droplets and the mitochondria appear blue, whilst the larger triglyceride drops are coloured red. The diffuse lipid noted in the cytoplasm of this cell also coloured with the acid haematein reaction, so it is presumably a phospholipid.

It is apparent that the fibrocytes of the two species of leech bear a great deal of structural resemblance to each other; there are, however, differences in their chemical constitution. The fibre-producing cell in *Hirudo* has large drops of triglyceride, a cytoplasm that has not a very low iso-electric point nd contains much acid mucopolysaccharide and diffuse phospholipid; that of *lossiphonia*, on the other hand, contains no triglyceride, but only phospholipid roplets and mitochondria, together with much RNA. The latter substance oes not seem to occur in the same type of cell in *Hirudo*.

THE GROUND-SUBSTANCE

In unstained sections and spreads of living tissue examined by phase-ontrast, the ground-substance appears perfectly homogeneous, though it nay be recognized in standard histological preparations by its coloration vith dyes such as light green and aniline blue. The ground-substance also hows a strong affinity for metallic ions, as was shown by Bensley (1934). In *irudo* it is found that fixation of the ground-substance is difficult; when oagulant fixatives such as Zenker's fluid are used the ground-substance no onger appears homogeneous but the basiphil material contained in it is found o have aggregated into small spherical droplets which alone colour and react o histochemical tests. It was found that this aggregation did not occur with ormaldehyde fixation, so that for all studies on fixed material this was used s the standard fixative.

The refractive index of the ground-substance was measured in a preparation f living connective tissue by use of the interference microscope. The value vas found to be 1·353, which agrees with the value of 1·35 found for the round-substance of the pond leech.

istochemistry

The ground-substance was found to have an M.B.E. value of pH 4, coniderably less than that of the fibres. No lipids could be detected in it, even fter all the unmasking procedures suggested by Ciaccio had been tried. here was a feeble reaction to the coupled tetrazonium test, no result with he Sakaguchi test, but a strong positive with the Hg nitrite test for tyrosine. he PAS reaction was positive, and there was strong metachromasy, which ould be suppressed by hyaluronidase treatment. The conclusion must be hat here again is a site of concentration of acid mucopolysaccharide. It is ossible that there is a neutral mucopolysaccharide present as well; but as the riginal metachromasy of this tissue is so intense, it is not possible to decide hether there is any increase on sulphation, and at present no conclusion an be reached on this point.

DISCUSSION

The most striking feature of this study is the general similarity between the wo species of leech in their connective-tissue fibres, fibrocytes, and ground ubstance. The similarities are perhaps most marked in the fibres: in both pecies they show the cortex and medulla, they run singly in the ground ubstance, and as far as can be ascertained by histochemical methods they are omposed of a collagen or a similar protein. The cells which secrete the fibres how minor differences, such as the presence of triglyceride fat drops in the fibrocytes of *Hirudo*. It seems probable that this is part of the adipose reserve of the body, for similar fat is found to accumulate in the cells of the crop wall. This difference is correlated with the absence, in *Hirudo*, of the adipose cells which form such a conspicuous feature of the connective tissue of *Glossiphonia*.

The other major difference is that *Hirudo* has relatively much more ground substance than the rhynchobdellid leech, and that the ground-substance has a much larger content of acid mucopolysaccharide. Increase in the amount of ground-substance may be supposed to be a consequence of the greater degree of coelom reduction found in the jawed leeches. It is also noticeable that the general body 'stiffness' of *Hirudo* is much less than that of *Glossiphonia*, a fact which was pointed out by Bourne (1884). This he attributed to differences in the amount of connective-tissue substance, but it may well be related in some way to the observed differences in the proportions of acid and neutral mucopolysaccharides. In this we may find some clue to the special functions of the connective-tissue ground-substance, such as direct control of the cellular environment, and it may well be that such activities are influenced by the presence of these two types of substance.

No observations made during the course of the present work throw any light on the process of fibrogenesis, especially on the question whether the cortex is intra- or extracellular. A further study of this tissue by means of the electron microscope has been undertaken in order to attempt to answer this question.

I wish to acknowledge my great debt to Dr. J. R. Baker, for his continued encouragement and helpful advice. My thanks are also due to Professor Sir Alister Hardy for continued facilities for research in his department, and to Dr. K. Little and Miss Maureen Westerdon for undertaking the X-ray diffraction study of the fibres. The work was carried out during the tenure of a Senior Hulme Scholarship of Brasenose College, Oxford, and of a grant from the Department of Scientific and Industrial Research.

REFERENCES

Aoyama, F., 1929. Z. wiss. Mikr., **46**, 489.
Astbury, W. T., 1938. Trans. Faraday Soc., **34**, 378.
Baker, J. R., 1944. Quart. J. micr. Sci., **85**, 1.
—— 1946. Ibid., **87**, 441.
—— 1947. Ibid., **88**, 115.
—— 1949. Ibid., **90**, 293.
—— 1956. Ibid., **97**, 161.
—— 1957. Ibid., **98**, 29.
Bensley, S. H., 1934. Anat. Rec., **60**, 93.
Bourne, A. G. 1884. Quart. J. micr. Sci., **24**, 419.
Bradbury, S., 1956. Ibid., **97**, 323.
—— 1957. Ibid., **98**, 29.
Cain, A. J., 1947. Ibid., **88**, 363.
Calleja, C., 1898. Z. wiss. Mikr., **15**, 322.
Champetier, G., and Fauré-Fremiet, E., 1938. J. chim. Phys., **35**, 223.
Chou, J. T. Y., 1957. Quart. J. micr. Sci., **98**, 47.

Ciaccio, C., 1926. Boll. soc. Biologia sper., **1**, 47.
Danielli, J. F., 1947. Symp. Soc. exp. Biol., **1**, 101.
Dempsey, E. W., Singer, M., and Wislocki, G. B., 1950. St. techn., **25**, 73.
Feulgen, R., and Rossenbeck, H., 1924. Z. phys. Chem., **135**, 203.
Gomori, G., 1952. *Microscopic histochemistry.* Chicago (University Press).
Herxheimer, G. W., 1901. Deut. med. Woch., **36**, 607.
Jackson, S. F., 1955. Nature, **175**, 39.
Jordan, B. M., and Baker, J. R., 1955. Quart. J. micr. Sci., **96**, 177.
Lankester, E. R., 1880. Ibid., **20**, 307.
Lison, L., 1953. *Histochimie et cytochimie animales.* Paris (Gauthier-Villars).
Pantin, C. F. A., 1948. *Microscopical technique.* Cambridge (University Press).
Pearse, A. G. E., 1954. *Histochemistry, theoretical and applied.* London (Churchill).
Pischinger, A., 1926. Z. Zellforsch., **3**, 169.
Ross, K. F. A., and Chou, J. T. Y., 1957. Quart. J. micr. Sci., **98**, 341.
Scriban, J. A., and Autrum, H., 1934. 'Hirudinea' in Kukenthal and Krumbach's *Handbuch der Zoologie.* Berlin (De Gruyter).
Weigl, R., 1910. Bull. Intern. Acad. Sci. Cracovie, Ser. B (no vol. no.), 691.
Wolman, M., and Greco, J., 1952. St. techn., **27**, 317.

APPENDIX

Results

Test	*Reference*	*Ground substance*	*Fibre*	*Fibrocyte cytoplasm*	*Large lipid drops*	*Small lipid drops*
Picro-indigo carmine	Calleja, 1898	blue	blue	orange	—	—
Masson . .	Pantin, 1948	green	green	reddish	—	—
Basiphilia . .	—	+	—	—	—	—
Acetic acid . .	—	—	swells and dissolves	—	—	—
Methylene blue extinction . .	Pearse, 1954	pH 4	pH 5	pH 4	—	—
Refractive index .	—	1·353	—	—	1·486	—
Aoyama . .	Aoyama, 1929	O	O	O	O	+++
Mann Kopsch .	Weigl, 1910	+	+	O	O	+++
Neutral red . .	—	O	O	O	O	+++
Methylene blue .	—	O	O	O	O	+++
Sakaguchi . .	Baker, 1947	O	++	+	O	—
Hg nitrite . .	Baker, 1956	+	O	+	O	—
Coupled tetrazonium	Pearse, 1954	O	++	++	O	—
C.T.+benzoylation	,,	O	+	O	O	—
C.T.+dinitrofluorobenzene . .	,,	O	+	O	O	—
C.T.+performic acid	,,	O	O	++	O	—
Sudan black . .	Baker, 1944, 1949	O	O	++ diffuse	+++	+++
Sudan IV . .	Herxheimer, 1901	O	O	O	+++	+++
Sudan black after Da Fano's fluid .	—	O	O	O	+++	O
Sudan black after phenol . .	Ciaccio, 1926	O	O	+ diffuse	+++	++
Sudan black after Cajal's fixative .	—	O	O	+ diffuse	+++	++

Results (cont.)

Test	*Reference*	*Ground substance*	*Fibre*	*Fibrocyte cytoplasm*	*Large lipid drops*	*Small lipid drops*
Sudan black after cold acetone	—	O	O	O	O	O
Nile blue	Cain, 1947	O	O	O	pink	—
Acid haematein	Baker, 1946	O	O	+	O	+++
„ control	„	O	O	O	O	O
Liebermann	Lison, 1953	O	O	O	O	—
Windaus	„	O	O	O	O	—
Pseudo-plasmal	„	O	O	O	++	—
Performic acid Schiff	Pearse, 1954	O	O	O	+++	—
PAS	„	++	+++	+++	+	—
PAS control	„	O	O	O	+	—
PAS after saliva	„	+	++	+++	+	—
Toluidine blue	„	+++	+	++	O	—
Toluidine blue+hyaluronidase	„	O	O	O	O	—
Feulgen	Feulgen and Rossenbeck, 1924	O	O	O	O	—
Pyronin / methyl green	Jordan and Baker, 1955	++	++	++	O	—
P/Mg saliva control	Bradbury, 1956	++	++	++	O	—
Gomori alkaline phosphatase	Gomori, 1952	O	O	O	O	—
Gomori acid phosphatase	„	O	O	O	O	—

Key: +++ Strong reaction.
++ Moderate reaction.
+ Weak reaction.
— No observation.
O Negative reaction.

A Study of Fibrogenesis in the Leech, *Hirudo medicinalis*

By S. BRADBURY *and* G. A. MEEK

(*From the Cytological Laboratory, Department of Zoology, and the Department of Human Anatomy, University Museum, Oxford*)

With four plates (figs. 1–4)

SUMMARY

The fibrocytes and the fibres of *Hirudo medicinalis* have been studied with the electron microscope. The fibrocyte can be shown by other methods to contain triglyceride droplets, phospholipid droplets, and mitochondria, all of which can be recognized in the electron micrographs. In addition, a 'vesicular' component has been observed just below the cell surface.

The collagenous 'cortex' of the fibre is tubular, and can be shown to contain a long process of the cell which secretes it. There are many small fibrils about 250 Å in diameter comprising the cortex of the fibre; they lie along the fibre axis, and often are associated in pairs or groups of three or four. These fibrils sometimes show a poorly defined periodicity of 300 Å.

It seems that the fibrils 'shred off' from the surface of the fibrocyte. The process shows some resemblance to that described in the chick by other authors.

ALTHOUGH the existence of fibres in the connective tissues of leeches has been known for a long time, little work has been published on them in the last twenty years. Recently these fibres have been studied structurally and histochemically in the leeches *Glossiphonia complanata* and *Hirudo medicinalis* (Bradbury, 1957, 1958).

The fibres appear to be composed of a 'cortex' and a 'medulla'; the latter seems to be a long process of the fibrocyte cytoplasm. It has been shown by means of histochemical tests and by X-ray diffraction studies that the cortex of the fibre is composed of collagen or a similar protein. No evidence was obtained from the previous work that would help to elucidate the question whether the fibre was secreted extra- or intracellularly, and whether the collagen of this invertebrate resembled that of vertebrates in possessing a submicroscopic periodicity. This last point is of some interest, as a report has been published describing the occurrence of unstriated collagen in the earthworm cuticle (Reed and Rudall, 1948), whilst, on the other hand, collagen obtained from certain cephalopod molluscs seems to be of the typical vertebrate structure (Schmitt, Hall, and Jakus, 1942).

The electron microscope has been used for many studies of fibrogenesis in vertebrates, particularly by Porter (1951), Wyckoff (1952), and Martin (1953). Very little attention has been paid to similar problems in invertebrates, however, and it is the purpose of this paper to give the results of a study of the fine structure of the fibres and fibrocytes of the leech *H. medicinalis*.

[Quarterly Journal of Microscopical Science, Vol. 99, part 2, pp. 143–148, June 1958.]

MATERIAL AND METHODS

Pieces of connective tissue were dissected out of the animal and placed immediately in ice-cold 1% osmium tetroxide solution with 0·22 M sucrose and 0·035 M veronal buffer at pH 7·3 (Caulfield, 1957). After dehydration in graded alcohols, the tissue was embedded in *n*-butyl methacrylate. Sections were cut on a Porter–Blum microtome; only those sections showing silver or gold interference colours were examined. After being mounted on Smethurst High-light 3-mm grids covered with a carbon film, the sections were studied in a Siemens Elmiskop I, operated at 60 kV.

The methacrylate was removed from some sections by immersing them overnight in redistilled ethylene dichloride, a technique suggested by Dr. Porter. These sections were then lightly shadowed with platinum before examination. In an attempt to obtain dissociated fibres, connective tissue from the leech was blended in a high-speed blender in the minimal quantity of formaldehyde solution for periods of several minutes. Drops of the resulting homogenate were dried down on to grids, shadowed with gold-palladium or platinum, and examined.

STRUCTURE OF THE FIBROCYTE

As shown by the light microscope, this cell is bipolar and roughly spindle-shaped, with two long processes extending from it for over 100μ on each side of the cell-body. From studies of the living cell by phase-contrast and interference microscopy, and from the histochemical studies already quoted, it is possible to list the cytoplasmic inclusions with some certainty.

Besides the nucleus, it is easy to detect the mitochondria, which are small, rod-shaped, and rather few in this cell. They are generally dispersed towards the periphery of the cell. The remaining two types of inclusion are both lipid in nature; one is a group of small lipochondria which are clustered round the nucleus and can be shown to contain phospholipid. The other type of lipid droplet is much larger (up to 3 μ in diameter) and very numerous. These fat droplets occupy most of the cytoplasm of the fibrocyte, and form the most conspicuous object in the cell; they seem to be composed almost entirely of triglyceride.

In the electron micrographs each of these types of cytoplasmic inclusion may be recognized (figs. 1; 2, A). The triglyceride droplets are very obvious, and it is noticeable that they appear badly shrunken, so that they have crenated edges. Studies of this type of droplet in living cells show them to be apparently perfectly spherical, so that this crenation is probably an artifact.

FIG. 1. (plate). A fibrocyte cut in longitudinal section.

FIG. 2 (plate). A, a fibrocyte cut in transverse section, showing all the cytoplasmic inclusions. Note the vesicular component just below the cell membrane.

B, a tangential section through the cortical region of one of the connective-tissue fibres; the small fibrils are clearly visible.

C, isolated fibrils from the connective tissue, showing some ill-defined surface periodicity. Shadowed with gold-palladium.

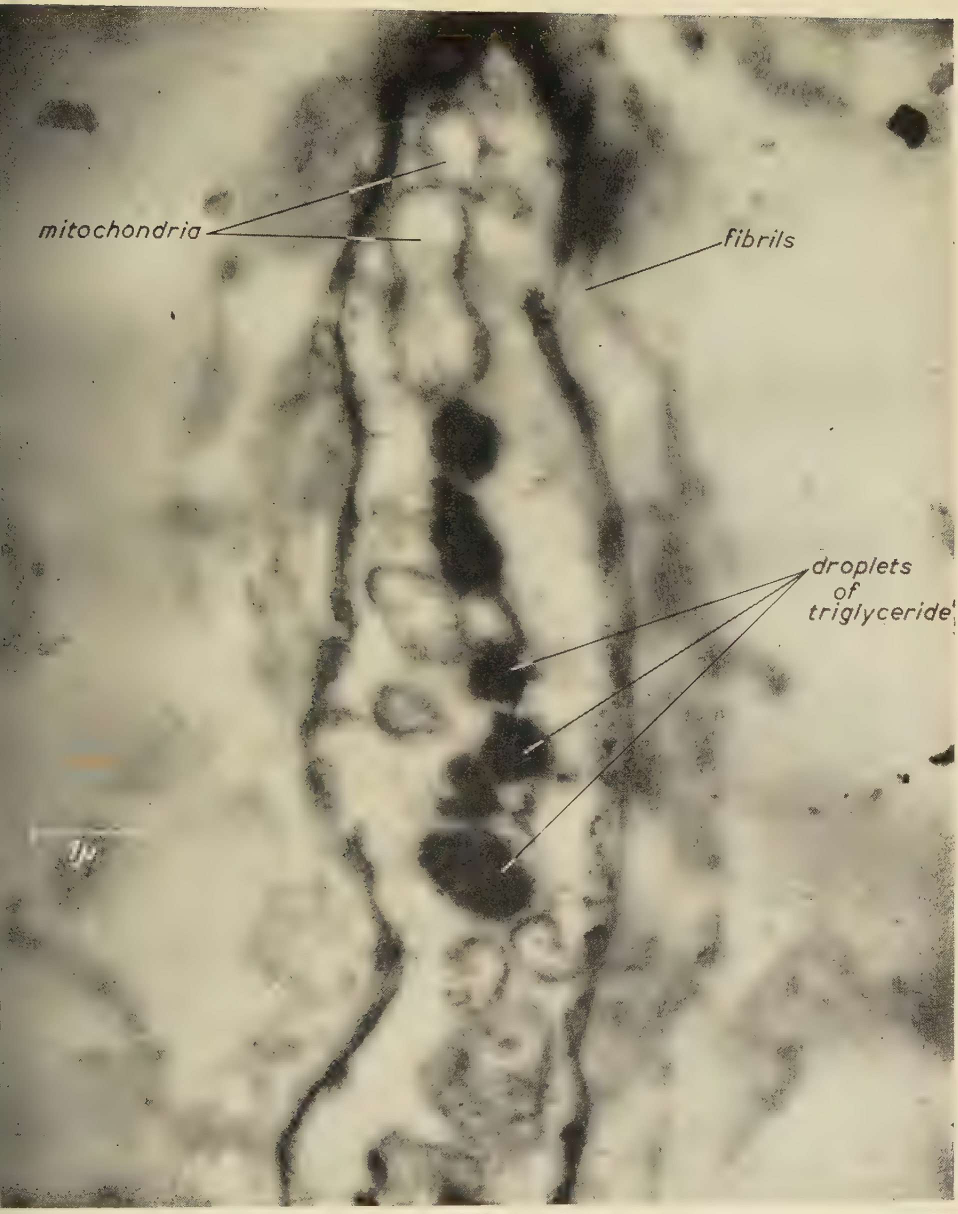

FIG. 1

S. BRADBURY *and* G. A. MEEK

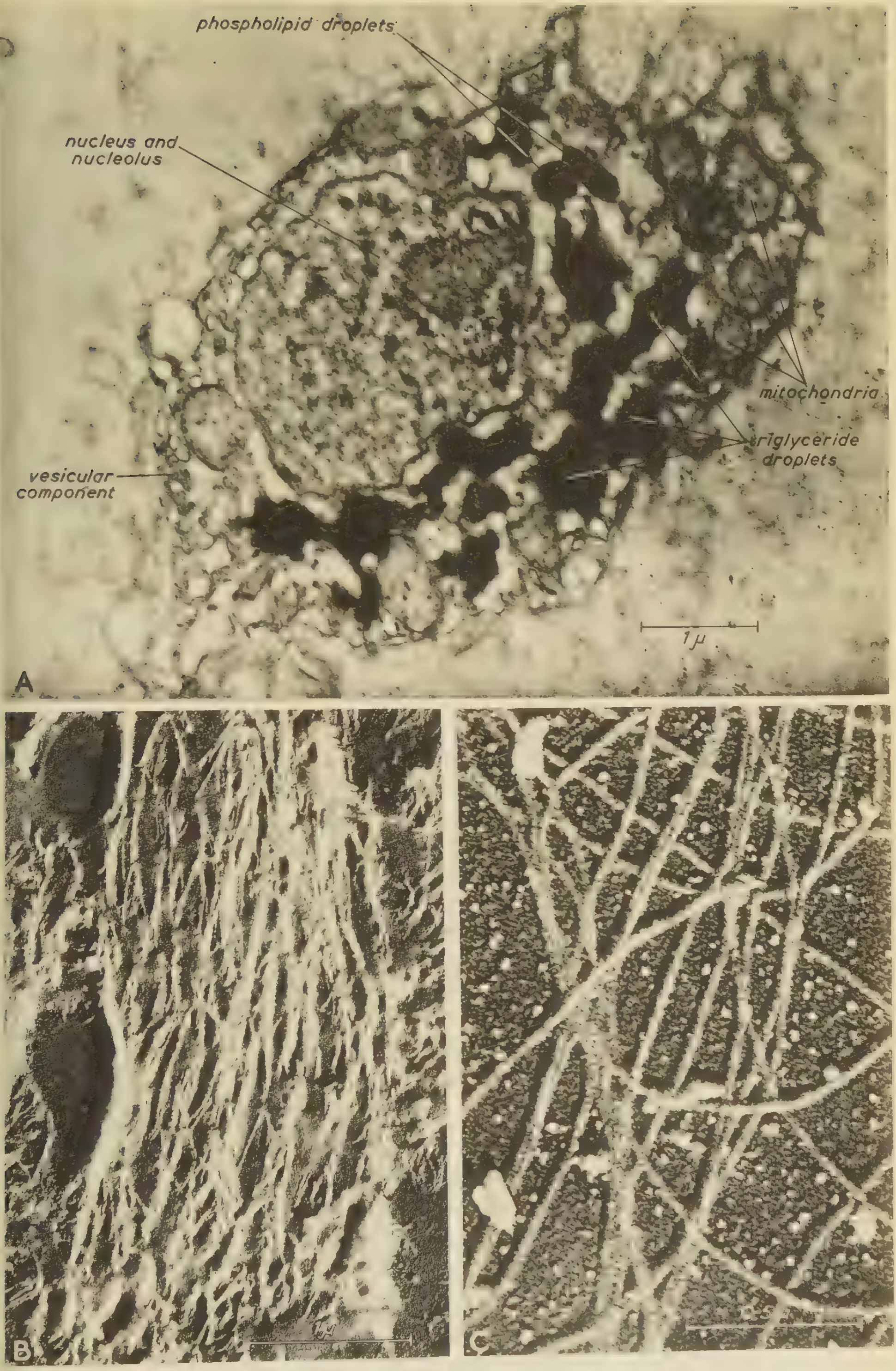

FIG. 2

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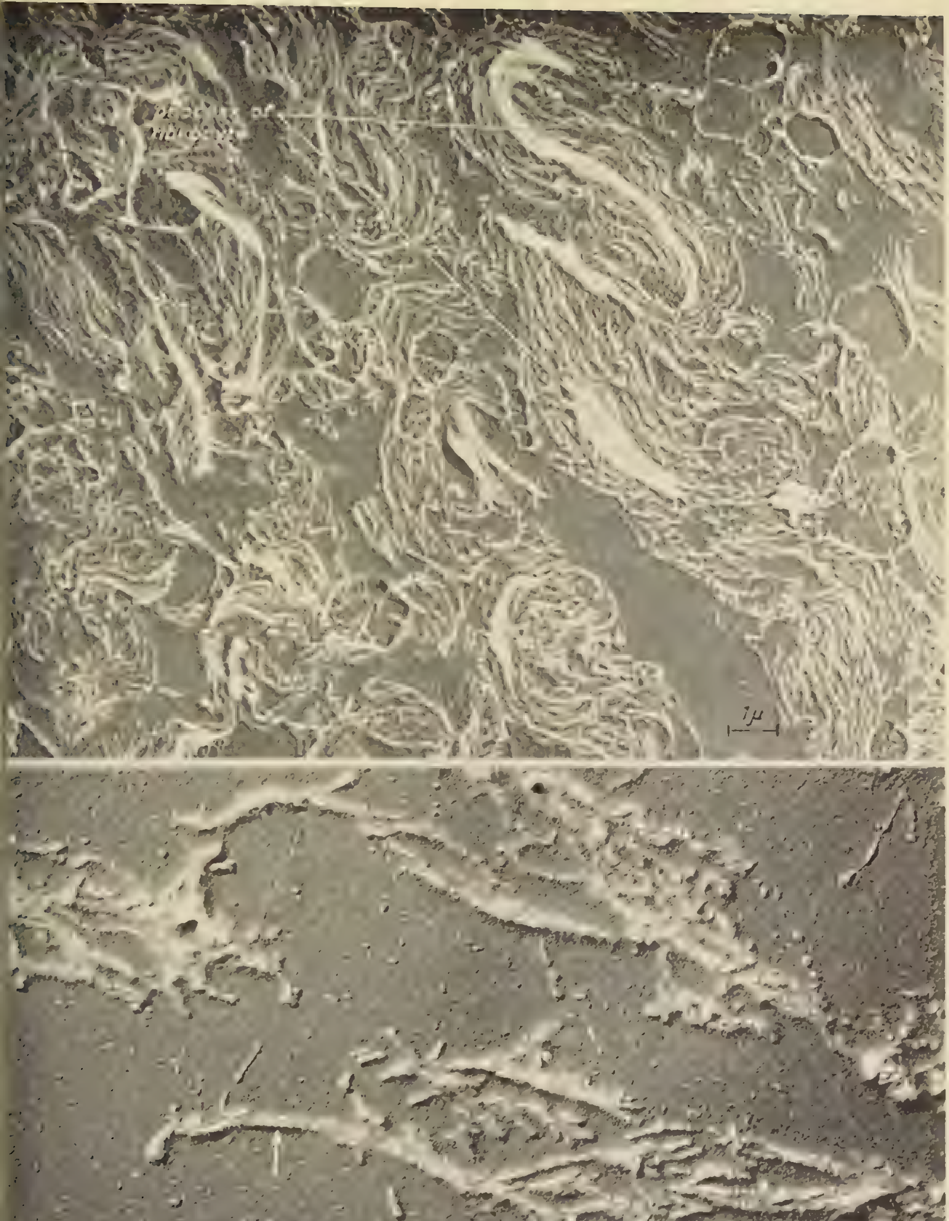

FIG. 3

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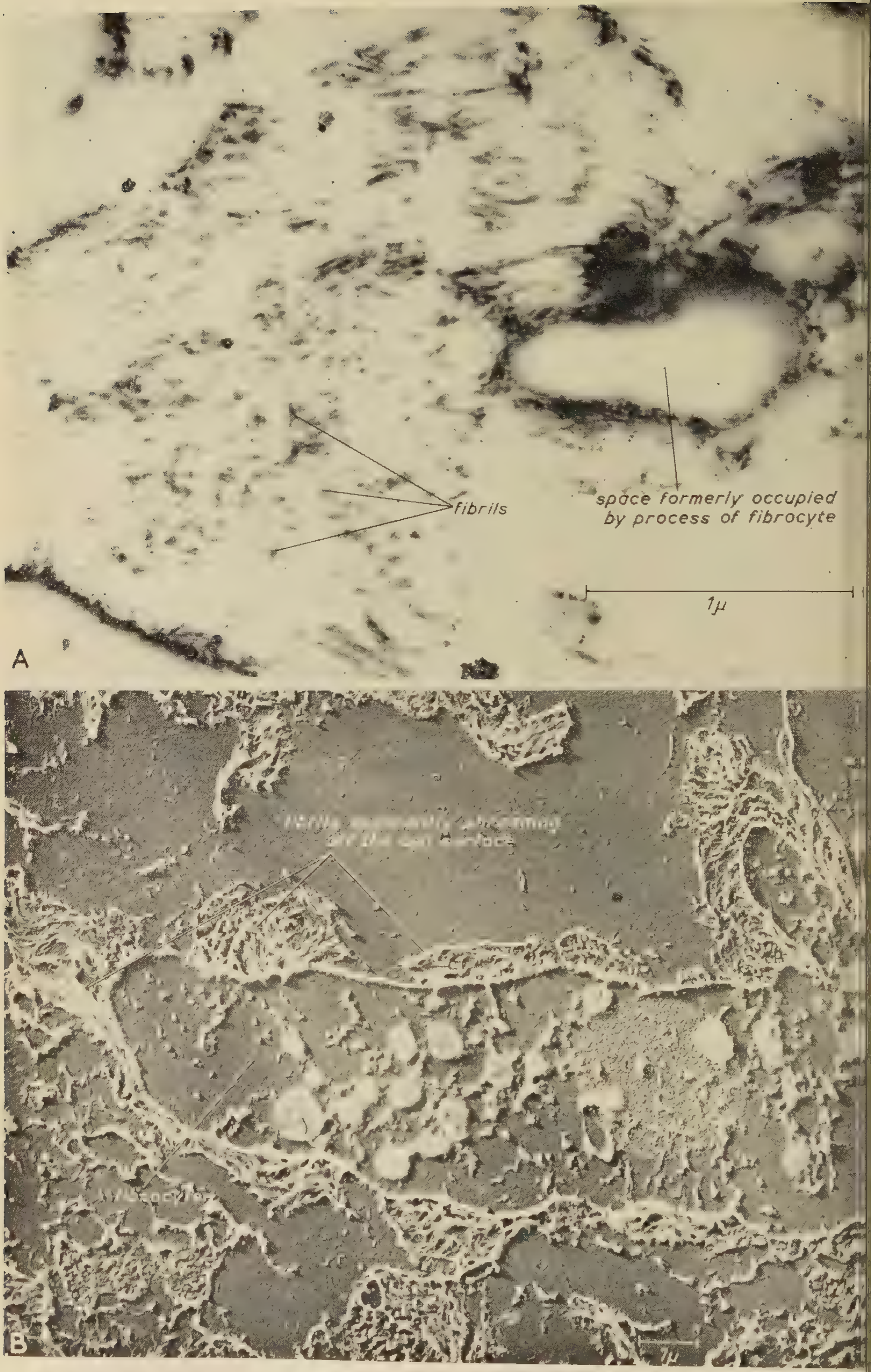
fibrils
space formerly occupied by process of fibrocyte
1μ
A
fibrils apparently emerging
on the cell surface
fibrocyte
1μ
B

FIG. 4

S. BRADBURY *and* G. A. MEEK

The triglyceride droplets are very electron-dense, probably because unsaturated lipid reduces the osmium tetroxide of the fixative.

Mitochondria are generally characterized in electron micrographs by the double membranes of the cristae; in the present material, however, it was found that the cristae were very poorly developed, or were poorly preserved by the technique of fixation used. It was possible to identify the mitochondria by comparing their shape, size, and position in the cell with those of mitochondria studied in living cells and in cells prepared with a standard mitochondrial technique.

The droplets which contain phospholipid are electron-dense, but less so than the triglyceride droplets. They are not greatly distorted by the fixation and embedding, but usually preserve the spherical shape seen in the living cell. No internal structure is visible, but there is some suggestion of an external membrane, which may be double.

One further cytoplasmic component is apparent in the electron micrographs which cannot be detected in either the living or the fixed cell, when studied with the light microscope. Immediately below the cell surface a complex of vesicles may be seen (fig. 2, A). It is not certain whether the vesicles seen in transverse section are isolated, or are continuous down the long axis of the cell. In one cell, where the plane of section was tangential to the cell surface, and just below it, the vesicles seem to be dilatations of a continuous system of canals or small tubes. This vesicular component of the fibrocyte cytoplasm will be considered further in the discussion.

THE STRUCTURE OF THE FIBRE

The ground substance of the leech connective tissue can be seen by light microscopy to contain numerous fibres, interlacing in all directions. When the connective tissue is examined under the lowest powers of the electron microscope a similar fibrous appearance may be seen, though as the sections are only about 200 Å thick, very little of each individual fibre may be visible (fig. 3, A). The fibres seemed to contain some sub-microscopic structure, so photographs were made at higher magnification. The actual collagenous material of the fibre forms the apparent cortex which is seen with the light microscope; it can now be seen to consist of numerous fibrils, approximately 250 Å in diameter, roughly orientated with their long axes parallel to the fibre axis. It appears from fig. 2, B that the fibrils associate in pairs or groups of three or four. It is probable that the space between the fibrils or groups of fibrils is occupied in life by the ground substance of the connective tissue.

FIG. 3 (plate). A, connective tissue of *Hirudo*, showing many fibres. In several places the process of the fibrocyte is apparent. Section shadowed with platinum.

B, connective-tissue fibrils; the arrow indicates two fibrils lying together, showing the periodicity. Shadowed with platinum.

FIG. 4 (plate). A, section through a single fibre. Note the space formerly occupied by the cell process, and the numerous fibrils, here mostly seen in tangential section.

B, fibrocyte, showing the fibrils apparently 'shredding' off the cell surface. The cytoplasmic inclusions are very poorly preserved. Section shadowed with platinum.

This would explain the positive periodic acid / Schiff reaction of the fibre which was noted in the previous paper (Bradbury, 1958).

It is not possible to measure the length of these fibrils in thin sections, so an attempt was made to isolate them by blending. Some fibrils found are shown in fig. 2, C, and from measurements of their diameter (about 300 to 500 Å) it seems that they represent not only individual fibrils, but also associated pairs. The fibrils are very long; the longest which it was possible to measure was over 3 μ in length and may well have been much longer in the living fibre. In fig. 2, B, C, which represent preparations lightly shadowed with platinum or gold-palladium, some suggestion is given of a surface periodicity or banding. Further sections, from which the methacrylate had been removed, were then soaked in distilled water for several hours in an attempt to remove any ground substance which might have been obscuring detail of the surface periodicity. The result is shown in fig. 3, B, where it is clear that the fibrils do show a poorly defined periodicity at an interval of 300 Å.

It was found that if tissue was fixed in 10% formaldehyde and subsequently embedded and sectioned in the normal manner, then only the fibrils of the fibre cortex were preserved, most of the fibrocyte cytoplasm and its inclusions being removed during dehydration and embedding. In one of these preparations a single fibre was seen in transverse section (fig. 4, A); again the association into small groups of fibrils is well marked, and particularly obvious is the space which in life would be occupied by the long process of the fibrocyte.

DISCUSSION

From consideration of the results described above, it seems reasonable to postulate that the fibrils which form the cortex of the connective-tissue fibre are actually produced at the cell surface. In sections from which the embedding medium has been removed and which have then been shadowed, this effect is very striking (fig. 4, B); the fibrils seem to shred off from the cell surface. A similar impression is obtained from pictures of fibre formation in the chick, which was studied by Wyckoff (1952), and fig. 8 in his paper is very similar to the appearance which is found in the present study. The material comprising the fibrils is presumably secreted just below the cell surface, and it seems reasonable to suppose that the 'vesicular' component noticed in the leech fibrocytes is active in this process. At present it is not possible to apply histochemical methods to the study of the levels of organization which are revealed by the electron microscope, so we are not able to form any more definite conclusions. It is interesting to speculate on the basis of the histochemical work on this cell which has been referred to previously. A striking feature was the presence in the cytoplasm of both diffuse and granular PAS-positive material which was also metachromatic, and of diffuse phospholipid. In view of the observations of Jackson (1955) that PAS-positive granules are extruded in chick fibrogenesis, it may be that in the leech fibrocyte the acid mucopolysaccharide is contained within the structure of the vesicular component, which may itself be primarily composed of phospholipid.

A further point of similarity between the process of fibrogenesis in this ech and in the chick is that in the latter, both Porter (1951) and Wyckoff 952) consider that the cell surface plays the most important role in organizg the fibrils of collagen outside the cell. On the basis of the present work, is possible to suggest that this is also true for the fibrogenesis of Hirudinea.

Although some periodicity has been detected in the fibrils composing the rtex of the connective tissue fibres of the leech, the periodicity is not the 40 Å banding which is characteristic of mammalian collagen, but a figure hich is about half of this. The periodicity of leech fibrils is never very well arked, in contrast to vertebrate material, and often it is not visible on all of e fibrils, but only on the thickest. The periodicity may, however, be masked y the presence of ground substance. The presence of periodicity in the fibrils a leech is rather interesting in view of the report by Reed and Rudall (1948) at the collagenous fibres of the earthworm cuticle are not striated. It is worth oting that the periodicity of 300 Å which is measured for these fibrils agrees ther closely with the periodicity of 270 Å which is found for some embryonic ick fibrils of approximately the same diameter (Martin, 1953; Porter, 1951). he significance of this observation is not apparent, but presumably it is ncerned with the degree of organization of the fibril at the molecular level. the vertebrates, stages of periodicity of 110 Å, 270 Å, and 640 Å are succesvely reached with increase in the diameter of the fibrils, whilst in the leech seems that as the maximum diameter of the fibril is about 300 Å, the eriodicity never attains the value found in the largest (and presumably the ost highly organized) mammalian fibrils. No fibrils were noticed with a eriodicity of less than 300 Å.

It seems then that in the leech *Hirudo*, the cortex of the fibre is collagenous nd surrounds the process of the fibrocyte which secretes it. This cell process emains inside the cortex throughout the life of the animal. In this work, and the previous study with the light microscope, fibres lacking the cellular rocess down their centre were never seen. Recently work has been published Vyckoff, 1952) which seems to show that some mammalian fibres are, in ct, hollow tubes. It may be that these mammalian fibres are secreted in a milar manner to those of the leech, but the cell and its process do not persist to adult life.

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REFERENCES

ADBURY, S., 1957. Quart. J. micr. Sci., **98**, 29.
— 1958. Ibid., **99**, 131.

Caulfield, J. B., 1957. J. biochem. biophys. Cytol., **3**, 827.

Jackson, S. F., 1955. Nature, **175**, 39.

Martin, A. V. W., 1953. *Nature and structure of collagen* (ed. Randall), p. 129. Londo (Butterworth).

Porter, K. R., 1951. *Proc. 2nd Jos. Macy Conference on Connective Tissues*, p. 126. New York (Jos. Macy Jnr. foundation).

Reed, R., and Rudall, K. M., 1948. Biochem. biophys. Acta, **2**, 7.

Schmitt, F. O., Hall, C. E., and Jakus, M. A., 1942. J. cell. comp. Phys., **20**, 11.

Wyckoff, R. W. G., 1952. *Proc. 3rd Jos. Macy Conference on Connective Tissues*, p. 38. New York (Jos. Macy Jnr. foundation).

A Morphological and Histochemical Study of the Oocytes of the Fish, *Ophiocephalus punctatus*, with particular Reference to Lipids

By H. C. CHOPRA

(*From the Department of Zoology, Panjab University, Hoshiarpur, India*)

SUMMARY

The cytoplasmic inclusions of the growing oocytes of the fish, *Ophiocephalus punctatus*, have been studied both morphologically and histochemically. It has been observed that the non-yolky oocytes of early stages consist of granules of two types, namely the mitochondria and the lipid granules of first category (L_1). The mitochondria are smaller granules, and they are found to be protein in nature with traces of lipids. The bigger granules (L_1) consist of phospholipids only; they correspond to the so-called 'Golgi bodies' of earlier workers on fish oocytes. The yolk found in the late oocytes is of two types. The first type of yolk globules, which appear with the approach of the breeding season, consist of lipids only, and have been described as lipid bodies of the second category (L_2). At first they are spheres consisting of phospholipids and triglycerides, but in the mature eggs they are transformed into big fat globules containing triglycerides only. The second type of yolk, which appears later, is in the form of spheres, rich in proteins and lipoproteins. Lastly there is 'vacuolar yolk', rich in carbohydrates and proteins; this develops in vacuoles.

INTRODUCTION

NATH and Nangia (1931) made an extensive study of the oocytes of the fish, *Ophiocephalus punctatus*, by employing osmium and silver techniques. They also made an extensive study of fresh oocytes under the light microscope. They contradicted the 'vacuome theory' of Parat and demonstrated clearly that the 'vacuome' was, chemically and functionally, a fundamentally different cell entity, independent of the osmiophil and argentophil 'Golgi bodies'. They reported that the vacuoles gave rise to albuminous yolk by condensing protein material in their interior, while the Golgi bodies were transformed into fatty yolk. Nath and others (see Nath, 1957) examined the living oocytes of the fish under phase contrast and fully confirmed the conclusions of Nath and Nangia (1931).

Very little work seems to have been done on the chemical nature of the contents of the fish egg, and only a few papers are available. Krishna (1950), while dealing with the histochemistry of lipids in the English trout, used Sudan black B, Sudan IV, and Nile blue sulphate, and demonstrated triglycerides, phospholipids, and lipoproteins in trout eggs. Recently Yamamoto (1955 *a*, *b*; 1956 *a–e*; 1957) has published a series of papers on the cytochemistry of fish eggs and has concluded that polysaccharides are found in the yolk formed in the vacuoles.

In this paper are recorded my observations on the morphology and chemical nature of the cytoplasmic inclusions of the egg of *O. punctatus* during their

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growth. The various histochemical techniques employed have demonstrated two types of granules in the earlier stages of oogenesis, viz. mitochondria and lipid granules of first category (L_1), which seem to give rise to yolk spheres and fat globules respectively. In addition to these, clear vacuoles are seen in the cytoplasm in earlier stages which give rise to the 'vacuolar yolk' containing a carbohydrate-protein complex, and do not show any traces of lipids.

MATERIAL AND TECHNIQUE

Specimens of the fish, *O. punctatus*, were collected from the ponds and ditches of Hoshiarpur. The ovaries from August to December were full of non-yolky oocytes; yolk formation begins in January or February only. After removal from the body the ovaries were first washed in saline solution (Baker, 1944). The tissue was then cut into very small pieces and transferred to various fixatives.

The material was fixed in Bouin, Champy, Lewitsky (Flemming without acetic), Kolatchev, Regaud, Helly, and Aoyama to demonstrate the morphology of the various cytoplasmic contents. Aoyama and Kolatchev preparations were examined unstained, but all other sections were stained with 0·5% haematoxylin after mordanting with 4% iron alum.

For histochemical study the material was mostly fixed in formaldehyde-calcium and postchromed according to the technique of Baker (1946). The fixed material was embedded in gelatin and the sections were cut at 10 μ. The various histochemical techniques employed for the location of lipids, carbohydrates, and proteins are given in tabular form in the Appendix.

The lipids were also studied by various solubility tests. The neutral fats were extracted with cold acetone. Cold ethanol and ether were used as general solvents for lipids. All these solvents were used for 24 h on the fresh material as well as on gelatin sections of material fixed in formaldehyde-calcium (without postchroming). Boiling ethanol was used as solvent for lipoproteins in the Soxhlet apparatus.

OBSERVATIONS

Morphological studies

In the earliest stages (fig. 1, A) numerous fine mitochondrial granules are seen in the cytoplasm, mostly close to the nucleus. They are coloured bluish by Sudan black B. Amongst this mass of fine granules lie a number of more prominent granules, which colour much more intensely with Sudan black and correspond to the Golgi bodies described by earlier workers on fish oocytes. They are here described as lipid bodies of first category (L_1), in accordance with their chemical nature, which will be dealt with in the later part of this paper. Soon the mass of mitochondria grows round the nucleus, forming a perinuclear ring (fig. 1, B). The mitochondria show linear alignment from now onwards so as to give the appearance of regular threads, which are coloured very clearly by Sudan black. These sudanophil threads are associated with a large number of strongly sudanophil L_1 granules. The mitochondria are not very well stained in these early stages by iron haematoxylin after chrome-osmium and Helly fixations, whereas the L_1 granules are conspicuously stained blue. In Aoyama / silver nitrate preparations also, only L_1 granules are blackened.

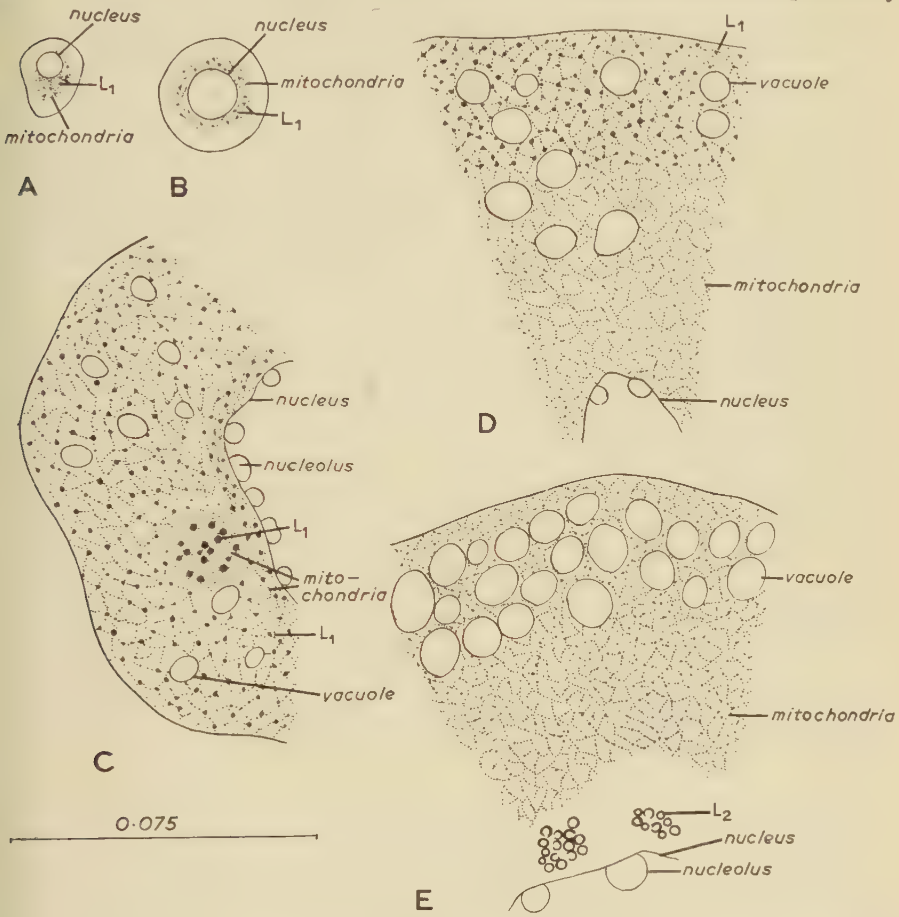

FIG. 1. Oocytes of *Ophiocephalus* fixed in formaldehyde-saline (A, B, C) and formaldehyde-calcium (D, E); all postchromed; the sections coloured with Sudan black B. A, earliest oogonial stage showing juxtanuclear concentration of mitochondria and L_1 granules. B, early oocyte showing perinuclear concentration of mitochondria and L_1 granules. C, a portion of an oocyte showing the distribution of mitochondria and L_1 granules in the entire cytoplasm, with a perinuclear and a local concentration of the cell inclusions. D, a portion of the oocyte showing two layers, the outer having mitochondria as well as lipid granules (L_1), while the inner contains only mitochondria. E, a portion of a well-developed oocyte, showing the complete absence of L_1 granules and the appearance of L_2 bodies in the clear perinuclear region.

Soon the mitochondrial threads of the perinuclear ring start spreading towards the periphery of the cell, carrying L_1 granules with them. This process continues until both kinds of cytoplasmic inclusions are nearly uniformly distributed in the cytoplasm (fig. 1, C). Even when the entire cytoplasm becomes occupied by these threads, some concentration of mitochondria and L_1 granules can still be observed in the perinuclear region. This perinuclear concentration appears very clearly under the phase-contrast microscope (see Nath, 1957, fig. 84, B). During such early stages, the mitochondria are seen as light-blue granular threads in chrome-osmium / iron haematoxylin preparations, but the L_1 granules are much more darkly stained. The latter bodies are blackened in Aoyama / silver nitrate preparations, but their granular appearance is revealed only after toning in gold chloride or by treating the silver with 4% iron alum. The L_1 granules are also blackened by the Kolatchev technique, but they require osmication for a very long period. However, the mitochondria appear as yellow threads in such preparations. In Bouin / iron haematoxylin preparations, only the mitochondria are preserved and stained blue, but they do not appear in the form of threads.

A large number of small vacuoles are also observed in the cytoplasm during the early stages. They are very clearly observed under the phase-contrast microscope and are stained with neutral red supravitally, as was shown by Nath and Nangia (1931). The enlarged vacuoles show a coagulum in their interior which is completely sudanophobe, osmiophobe, and argentophobe.

Although Sudan black invariably colours the spherical group of mitochondria and L_1 granules in the early oocytes near the nucleus (fig. 1, C), yet this area is not very clearly differentiated in chrome-osmium or silver nitrate preparations, though a concentration of argentophil bodies has occasionally been observed in some of the oocytes. This structure is very conspicuously observed in living oocytes under phase contrast; and Nath and his collaborators have photomicrographed it in the fish *Cirrhina* (see Nath, 1957, fig. 85, C). As the oocytes grow this concentration completely disappears from view.

The oocyte now enters a stage when the perinuclear concentration of mitochondria and L_1 granules moves towards the periphery and ultimately comes to lie beneath the epithelium, forming a peripheral layer (fig. 1, D). The L_1 granules of the perinuclear ring are often arranged in granular rods in the peripheral region before they are uniformly dispersed. The cytoplasm now exhibits two definite layers, the outer having a dense gathering of both types of granules and the inner consisting of mitochondria only. All the L_1 granules show an increase in size and appear as very prominent granules, whereas only some of the mitochondrial granules show growth.

The vacuoles show progressive increase in size along with the growth of the oocyte and move towards the periphery. At the same time the vacuolar yolk appearing in the vacuoles seems to become denser. The nucleus also grows with the increase of the cytoplasm. In some fixed preparations nuclear pouches containing nucleoli are observed; but, since I have never seen these in the living material, it is to be concluded that the pouches are artifacts due to the drastic paraffin technique, as shown earlier by Nath and his collaborators (1944).

The next stage of oogenesis is characterized by the uniform dispersal of L_1 granules throughout the cytoplasm and a slight increase in their size. The behaviour of mitochondria and L_1 granules towards osmium and silver echniques remains unchanged.

With the approach of the breeding season the oocytes seem to undergo a great physiological change. Several well-developed oocytes have been observed t this stage which showed complete lack of L_1 bodies, the whole of the oocyte being filled up with mitochondria, except a clear perinuclear region. Soon a number of collections of spherical bodies make their appearance in the clear perinuclear region (fig. 1, E). As these bodies, like L_1 bodies, have been shown by histochemical tests to consist of lipid only, they are here designated as ipid bodies of second category (L_2). These spheres are strongly osmiophil and argentophil. Most of these L_2 bodies do not colour homogeneously with Sudan black B, but appear as rings and crescents. The cortex of such bodies s strongly sudanophil, while the internum remains uncoloured. They are, however, homogeneously stained in chrome-osmium / iron haematoxylin preparaions. They blacken in Kolatchev and silver nitrate techniques. In Bouin / iron haematoxylin preparations the L_2 bodies are completely absent and only the mitochondria are observed. The mitochondria in these late oocytes show an ncrease in size, but they still show a linear arrangement. The vacuolar yolk fails to stain in chrome-osmium / iron haematoxylin and silver nitrate techniques and appears yellowish as in earlier stages. It is sudanophobe.

With the growth of the egg, the L_2 spheres grow rapidly so as to form big oval fat-globules, which spread towards the periphery (fig. 2, A, B). The fully formed L_2 bodies are blackened with osmium tetroxide in fresh cover-slip preparations. When chrome-osmium / iron haematoxylin preparations are heated after mounting in Canada balsam, the osmicated globules are decolorized and appear as empty vacuoles. They do not show any impregnation with silver.

In the very late oocytes, when the L_2 bodies have been transformed into arge fat-globules and the formation of vacuolar yolk has almost been completed, a large number of tiny spheres rich in protein matter are seen arising amidst the mitochondria at the periphery of the oocyte (fig. 2, A). Like L_2 bodies these spheres show rapid increase in size and number to form a large number of big yolk spheres. As these yolk bodies are very rich in proteins, they are here designated as protein yolk. The protein yolk fills up the entire cytoplasm in the fully developed eggs.

The protein yolk gives bluish coloration in Sudan black B. It behaves like L_2 yolk bodies with osmium and silver techniques, but is resistant to Bouin and also fails to blacken with Kolatchev.

Histochemical studies

The mitochondria are very fine granules in the early stages, which are aligned and appear as granular threads. The mitochondrial granules seem to be embedded in a thin ground-substance, owing to which they appear as continuous filaments. As this ground substance is strongly sudanophil, and is dissolved out with various fat solvents at room temperature, it seems to be purely lipid in nature. The mitochondrial granules are coloured blue with Sudan black B. In sections stained by Baker's acid-haematein technique the mitochondria in the early stages could not be differentiated from the pale yellow cytoplasm, probably because of their very small size; but in the late stages they could be recognized as blue granules. However, they were seen clearly as blue granules in the earlier as well as in the later stages in the pyridine-extracted control sections, which suggests that the blue coloration of mitochondria in the former preparations was not due to phospholipids. The presence of proteins in the mitochondria is revealed by their deep blue staining by the Hg-BPB test. The mitochondria give negative results with Nile blue (Cain, 1947), Sudan III and IV (Kay and Whitehead, 1941), Sudan IV, used as colloidal suspension in 1% gelatin, and Fettrot (Ciba): this suggests that they are devoid of neutral fats. They are also negative to Fischler's test for fatty acids. The mitochondria are negative to PFAS technique.

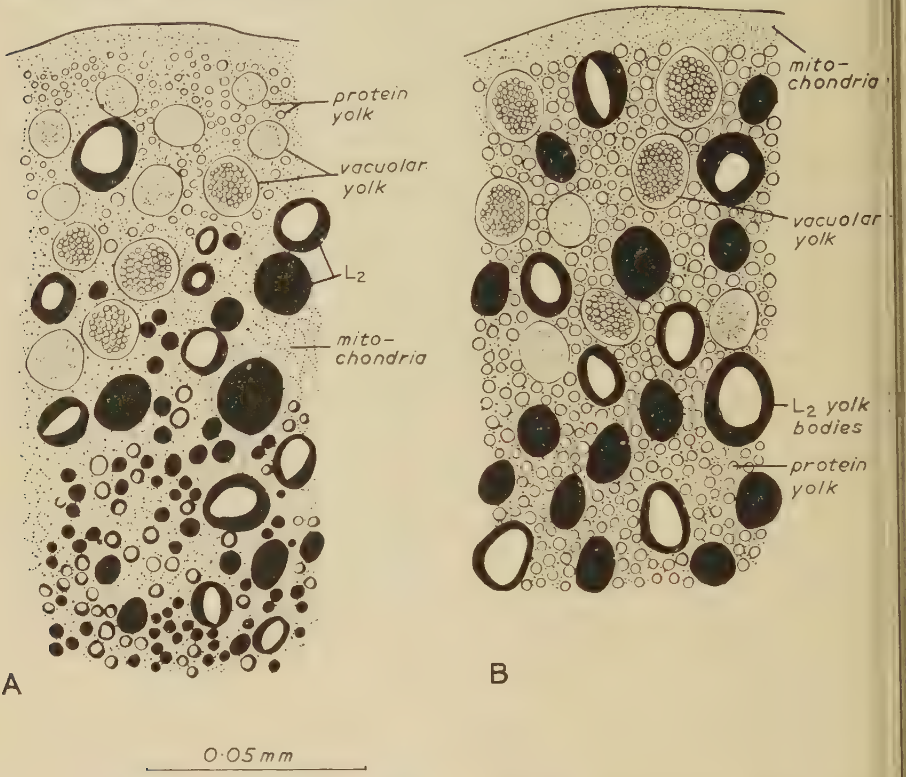

FIG. 2. Portions of very late oocytes. Formaldehyde-calcium; postchromed; Sudan black B. A, a stage showing 'vacuolar yolk', dispersal of L_2 yolk bodies towards the periphery, and the origin of protein yolk. B, almost mature egg showing fully grown 'vacuolar yolk', L_2 yolk bodies, and protein yolk bodies.

The mitochondrial granules show resistance to extraction by cold ethanol; but the ground-substance is completely dissolved out, with the result that they appear as scattered granules after this treatment. As the mitochondria colour blue with Hg-BPB even after treatment with hot ethanol, they probably contain some free protein.

The lipids of the first category (L_1 granules) are coloured darkly with Sudan black B. Unlike the mitochondria, they are AH-positive but negative after pyridine extraction, which shows that they contain phospholipids. But, like the mitochondria, they do not give positive results with the techniques employed for the demonstration of neutral fats. As the L_1 granules are coloured blue and not pink with Nile blue, they are acidic. The absence of fatty acids is shown by their negative reaction to Fischler's test. Their completely negative reaction to the Hg-BPB test for proteins and to the PAS technique for carbohydrates leads to the conclusion that the L_1 granules are purely lipid in nature, which is further confirmed by their complete solubility in ethanol and ether at room temperature.

The L_2 bodies are also strongly sudanophil, but do not colour homogeneously with Sudan black B at room temperature: they appear as rings and crescents. The cortex of these bodies is strongly sudanophil, while the internum is sudanophobe. Such an appearance of the L_2 bodies is seen even in the very late stages (fig. 2, B). Like the L_1 granules, the L_2 bodies in their early stages are AH-positive but negative after pyridine extraction; this shows the presence of phospholipids. These AH-positive sudanophil bodies of early stages give violet coloration with Nile blue. The presence of neutral fats was also suggested by their colouring red with Sudan III and IV and with Fettrot (Ciba), and orange-red with Govan's Sudan IV colloidal suspension.

However, when the L_2 bodies are fully grown, they seem to consist of neutral fats only, their phospholipid contents having completely disappeared. This is strongly indicated by their negative reaction to AH, their pink coloration (instead of violet) in Nile blue, their deeper coloration by the red Sudan colouring agents and by Fettrot, and their quick blackening in osmium tetroxide.

The L_2 bodies of earlier as well as later stages are completely washed out by acetone, ether, and ethanol at room temperature. After this treatment they appear as vacuoles. Their reaction to Fischler's test for fatty acids is negative. They colour pink with PFAS test, which shows that the contents of the L_2 bodies are unsaturated. This was also suggested by their quick blackening in osmium tetroxide in fresh coverslip preparations. Further, the L_2 bodies, like the L_1 bodies, do not have any proteins or carbohydrates in them, as is shown by the completely negative reaction to Hg-BPB and PAS respectively.

The protein yolk bodies, which make their appearance amongst the mitochondria, are in the form of numerous spheres. As they chiefly consist of proteins they are named as protein yolk bodies. Unlike the L_2 bodies, the protein yolk gives only a blue coloration with Sudan black B. Their coloration by AH is positive, but is not changed in the pyridine control sections. The reaction of the lipid contents of the protein yolk is negative to various tests for neutral fats, i.e. Nile blue and the red Sudan colouring agents. Similarly their negative results with Fischler's test shows the absence of fatty acids in them. As the sudanophil contents of the protein yolk are resistant to cold ethanol and ether, but are removable by hot ethanol, it is apparent that the lipid contents of this yolk are not in the free form but are in close combination with protein.

The presence of the protein component in this yolk is revealed by their deep blue coloration with Hg-BPB. Even after treatment with hot ethanol, these bodies colour deep blue in Hg-BPB. The negative reaction of the protein yolk to PAS shows the absence of carbohydrates in them.

Cholesterols and their esters are absent from the oocytes.

The vacuolar yolk that appears in the growing vacuoles of the oocytes is sudanophobe, unlike the yolk bodies just described. This yolk gives strongly positive coloration with the PAS technique, with negative results after the usual control of acetylation. The conclusion that it is very rich in carbohydrates is confirmed by the positive result after acetylation control and exposure to KOH. This yolk also contains some protein as well as carbohydrates, as it is lightly stained by the Hg-BPB test. Some of these yolk bodies have a frothy appearance.

DISCUSSION

It is clear from the descriptions given in the earlier part of this paper that there are five distinct kinds of cytoplasmic inclusions in the oocytes of *O. punctatus*, as follows:

1. *Mitochondria.*
2. *Lipids of the first category* (L_1), consisting of phospholipids only.
3. *Lipids of the second category* (L_2), consisting at first of phospholipids and triglycerides, later of triglycerides only.
4. *Protein yolk*, consisting of protein with some lipoprotein.
5. *Vacuolar yolk*, rich in carbohydrate, with some protein.

In very young oocytes the L_1 granules are closely associated with the mitochondria. They may perhaps originate from mitochondria.

The L_2 globules correspond with the 'fatty yolk' of Nath and Nangia (1931). In many animals the fatty yolk is derived from L_1 granules or so-called 'Golgi bodies' (see Nath, 1957, for full references), but this is not so in *Ophiocephalus*. After fixation in formaldehyde-calcium and colouring with Sudan black (Baker, 1946), the L_2 globules appear in the form of 'rings' and 'crescents'.

The protein yolk arises among the mitochondria. A transformation of mitochondria into 'albuminous yolk' has been described by Nath (1931) in the oocyte of the frog and by Nath and Malhotra (1954, 1955) in the oocytes of the frog and toad. Direct transformation of mitochondria into protein yolk was not observed in *Ophiocephalus* by Nath and Nangia (1931), and I have ot observed it myself. Nath and his collaborators (1944) have studied protein olk in the oocytes of many species of fishes without finding evidence of irect origin from mitochondria.

The vacuolar yolk corresponds with the yolk said by Yamamoto (1955 *a*, *b*; 956*a–e*; 1957) to be formed in 'vesicles'.

I take this opportunity of expressing my gratitude to Professor Vishwa Nath or suggesting this line of research, for his special interest, for encouragement uring the course of these investigations, and for correcting the manuscript. am also grateful to B. L. Gupta for some useful suggestions.

REFERENCES

AKER, J. R., 1944. Quart. J. micr. Sci., **85**, 1.
— 1946. Ibid., **87**, 409.
— 1949. Ibid., **90**, 293.
RADBURY, S., 1956. Ibid., **97**, 499.
AIN, A. J., 1947. Ibid., **88**, 383.
— 1948. Ibid., **89**, 429.
— 1950. Biol. Rev., **25**, 73.
LICK, D., 1949. *Techniques of histo- and cytochemistry*. New York (Interscience).
AY, W. W., and WHITEHEAD, R., 1941. J. Path. Bact., **53**, 279.
RISHNA, D., 1950. Proc. Nat. Acad. Sci. India, **20**, 6.
OVERN, J. A., 1955. *The chemistry of lipids of biochemical significance*. London (Methuen).
IAZIA, D., BREWER, P., and ALFERT, M., 1953. Biol. Bull., **104**, 57.
ATH, V., 1931. Z. Zellforsch., **13**, 82.
— 1957. Res. Bull. Panj. Univ. India, **98**, 145.
— GUPTA, B. L., and BHANGO, J. S. Unpublished, quoted from Nath, 1957.
— and MALHOTRA, S. K., 1954. Res. Bull. Panj. Univ. India, **59**, 149.
— — 1955. Ibid., **68**, 39.
— and NANGIA, M. D., 1931. J. Morphol. Physiol., **52**, 277.
— SINGH, B., and BAKR, A., 1944. Proc. Nat. Inst. Sci., **10**, 247.
EARSE, A. G. E., 1954. *Histochemistry*. London (Churchill).
AMAMOTO, K., 1955*a*. Annot. Zool. Japon., **28**, 158.
— 1955*b*. Ibid., **28**, 233.
— 1956*a*. Jap. J. Zool., **11**, 567.
— 1956*b*. Fac. Sci. Hokkaido Univ., **12**, 391.
— 1956*c*. Annot. Zool. Japon., **29**, 91.
— 1956*d*. Embryologia, **3**, 131.
— 1956*e*. J. Fac. Fish. Hokkaido Univ. **1**, 208.
— 1957. Annot. Zool. Japon., **30**, 33.

APPENDIX

Summary of histochemical tests used and results obtained

Test applied	*Fixation*	*Reference*	*Mitochondria*	*Lipids of first category (L_1)*	*Lipids of second category (L_2)*		*Protein yolk*	*Vacuolar yolk*
					Early	*Late*		
Sudan black B	Fca and PC	Baker, 1946	+	+++	+++	+++	+	O
	Fsa and PC	Baker, 1949	+	+++	+++	+++	+	O
Sudan black B* cold ethanol	Fr, Fca and WPC	Krishna, 1950	+	O	O	O	+	O
Sudan black B* cold ether	Fr, Fca and WPC	Krishna, 1950	+	O	O	O	+	O
Sudan black B* cold acetone	Fr, Fca and WPC	Krishna, 1950	+	+++	O	O	+	O
Sudan black B* hot ethanol	Fr	Krishna, 1950	O	O	O	O	O	O
Sudan black B* pyridine	WB and PE	Baker, 1946	+	O	O	O	+	O
Sudan III and IV (Kay and Whitehead, 1941)	Fca and PC	Pearse, 1954	O	O	++	+++	O	O
Sudan IV colloidal suspension (Govan, 1944)	Fca and PC	Pearse, 1954	O	O	++	+++	O	O
Fettrot (Ciba)	Fca and PC	Pearse, 1954	O	+	+++	+++	O	O
Nile blue	Fca and PC	Cain, 1947, 1948	O	O	++ (violet)	+++	O	O
Nile blue* cold acetone	Fr, Fca, WPC	Krishna, 1950	O	O	O	O	O	O
Acid-haematein (AH)	Fca and PC	Baker, 1946	++	+++	+++	O	+++	O
Acid-haematein* pyridine (PE)	WB and PE	Baker, 1946	+++	O	O	O	+++	O
Performic acid / Schiff (PFAS)	Fca and PC	Pearse, 1954	O	O	++	+++	O	O
Fischler's	F	Pearse, 1954	O	O	O	O	O	O
Schultz's	Fca and PC	Pearse, 1954	O	O	O	O	O	O
Mercuric bromophenol blue (Hg-BPB)	Fca and PC, B, Z	Mazia and others, 1953	+++	O	O	O	+++	+
Mercuric bromophenol blue* hot ethanol	Fca and PC, B, Z	Mazia and others, 1953. Glick, 1949	+++	O	O	O	+++	+
Periodic acid / Schiff (PAS)	Fca and PC, Z	Pearse, 1954	O	O	O	O	O	+++
PAS, control acetylation	Fca and PC	Pearse, 1954	O	O	O	O	O	O
PAS, control KOH	Fca and PC	Pearse, 1954	O	O	O	O	O	+++

KEY: +++ = strong reaction; ++ = moderate reaction; + = weak reaction; O = negative reaction; * = after treatment with; F = formalin; Fca = formaldehyde-calcium; Fsa = formaldehyde-saline; Fr = fresh tissue; WB = weak Bouin; PC = postchromed; WPC = without postchroming; B = Bouin's fluid; Z = Zenker

Histochemical Observations on the Cortical Region of the Oocytes of *Rana pipiens*

By R. M. ROSENBAUM

rom the Biological Laboratories, Harvard University, and Department of Pathology, Albert Einstein College of Medicine, New York 61, *N.Y.*)

With one plate (fig. 3)

SUMMARY

The cortical cytoplasm of the developing oocyte of *Rana pipiens* has been investigated y cytochemical means. Particular emphasis is placed on the nature and distribution of olysaccharide protein complexes. With toluidine blue O, both living and fixed ature oocytes were found to possess a distinct metachromatic region just under the eveloping vitelline membrane. Younger oocytes, at the previtelline-stage, did not exibit metachromasia in this region. Comparative studies suggested a similarity of ocyte cortical metachromasia to that of mucoitin sulphuric acid. Oxidation by periodic id and staining with the Schiff reagent or 2,4-dinitrophenylhydrazine or both, emonstrated reactive granules located in the cortex of both young and mature oocytes. he presence of more than one polysaccharide component was suggested. Hydrolysis ith trypsin and chymotrypsin indicated that the proteins in the ectoplasmic region ere more readily digestible than those in the endoplasm.

THE cortical region of the amphibian egg has been shown to have considerable significance during fertilization (Allen and Hagstrom, 1955), in ell division (Selman and Waddington, 1955; Schectman, 1937), and in the asic polarity of the egg and the *potential morphogénetique* (Dalcq, 1940). In mphibian development Holtfreter (1943, 1948) has described an elastic surace coat that he considers responsible for many fundamental phenomena such s cell permeability, osmotic regulation, movement, and aggregation. It is ear that this elastic layer consists of the cortex itself or some product derived om it. A fuller understanding of the structure and functions of the cortex of gg cells is thus important in order to acquire a fuller grasp of morphogenesis. n the present paper, cytochemical observations on the cortex of the oocyte of *ana pipiens* are reported which provide additional information as to the rganization of this region.

MATERIAL

The female *R. pipiens* used in this study were obtained from a commercial ealer in Vermont, U.S.A., and from ponds in the vicinity of Cambridge, assachusetts. Ovaries were removed from the animals during July and ugust and placed in cool amphibian Ringer's solution, in which individual ocytes with their follicular envelopes were dissected out. These were rranged in stages according to a system described in detail elsewhere (Rosenaum, 1953) and summarized in table 1. Those preparations that were fixed

uarterly Journal of Microscopical Science, Vol. 99, part 2, pp. 159–169, June 1958.]

were dehydrated in ethanol and cleared in cedarwood oil or dehydrated and cleared in dioxan, infiltrated with paraffin at 54° to 56° C under vacuum, and sectioned at 6 to 10 μ.

TABLE 1

Stage criteria for the oocytes of R. pipiens

Stage	*Oocyte size* (μ)	*Ooplasm (basiphilia)**	*Yolk*	*Pigment*	*Germinal vesicle*
I	below 200	intense	none visible	none visible	none
	200–400	less intense	ditto	ditto	development of nuclear membrane
II	450–650	little in endoplasmic region	appearing in yolk 'nucleus' and periphery	yellow brown	enlargement of nuclear membrane, sacculation
III	650–800	only in cortex	definite polar distribution	brown to black	no change
	800 to 1·8 mm	not easily demonstrable in cortex	ditto	ditto	maximum size

* As determined by the May–Grünwald technique.

EXPERIMENTS AND RESULTS

Metachromasia

In preliminary experiments, fixed oocytes at stage III were stained while in dilute solutions of metachromatic dyes, such as toluidine blue O (TBO), thionine, celestin blue B, and pinacyanole. A region just under the vitelline membrane exhibited a metachromatic colour. This staining property was then studied in unfixed oocytes of all stages.

Experiment 1. Fresh, unfixed oocytes were placed for varying periods in different concentrations of TBO made up in amphibian Ringer's solution at pH 6·8. The NaCl in this solution prevented formation of an inactive mucin complex. After exposure to the dye, the oocytes were examined under a binocular dissecting microscope for the shade of staining. The observations are presented in table 2.

At least three factors appear to influence the final colour response of living oocytes to TBO: dye concentration, length of exposure, and diffusion. The mature (stage III) oocyte is a cell approximately 1·7 mm in diameter with a fully formed vitelline membrane, and under these circumstances it is impossible to be sure what factors control diffusion. The stripped younger oocytes (stages I and II) are considerably smaller in diameter and have only the ooplasmic membrane as a barrier to diffusing substances. The metachromatic shades (red purple and purple) appeared only when the oocytes were exposed to low concentrations of the dye for short periods and then only in the cortical region just beneath the ooplasmic or vitelline membrane. In instances where deep penetration of the dye occurred, the interior of the oocyte always exhibited the orthochromatic shade of the dye.

TABLE 2

Colour uptake by fresh oocytes exposed to graded concentrations of toluidine blue O for varying periods. The dye was made up in isotonic amphibian Ringer's solution, pH 6·8

		Stage I oocyte (unpigmented)		*Stage II and III oocytes (pigmented)*	
TBO concentration	*Exposure time* (min)	*Cortex*	*Interior*	*Cortex*	*Interior*
$1:1\times10^{-3}$ . .	30	l. blue	no colour	l. blue	no colour
	60	d. blue	l. blue	d. blue	no colour
	120	d. blue	d. blue	d. blue	l. blue
$1:1\times10^{-4}$ . .	30	l. blue	no colour	l. blue	no colour
	60	l. blue	l. blue	l. blue	no colour
	120	d. blue	l. blue	d. blue	l. blue
$1:5\times10^{-4}$ . .	30	l. blue	no colour	l. blue	no colour
	60	l. blue	l. blue	d. blue	no colour
	120	d. blue	l. blue	d. blue	no colour
$1:1\times10^{-5}$ . .	30	purple	no colour	red purple	no colour
	60	d. blue	no colour	red purple	no colour
	120	d. blue	l. blue	blue purple	no colour

The relation of dilution to the production of metachromasia in terms of the chromotrope or chromotropes producing the colour was next investigated.

Experiment 2. Samples of mouse-tail cartilage, frog xyphisternum, human umbilical cord, and mouse and frog small intestine were fixed together with frog oocytes in cold 4% basic lead subacetate for 24 h. These were washed well in tap-water, hardened in 10% neutral buffered formalin for 12 h, run up to paraffin, and sectioned. One drop of an aqueous stock solution of TBO (1:800) was added per 100, 90, 80, 70, 60, 50, 40, 30, 20, or 10 ml of distilled water. The sections were placed together in these dye solutions at 53° C for 24 h. At the end of this period the sections were washed in tap-water and the colour was evaluated against an arbitrary colour standard.

Mouse and frog cartilage and the Wharton's jelly of human umbilical cord are known to contain large amounts of chondroitin sulphate, while the mucous goblet cells of mouse and frog small intestine contain mucoitin sulphate. By this staining method, chondroitin components stained with TBO at lower dilutions than did mucoitin elements. In fig. 1 it can be seen that the cortex and vitelline membrane of the oocyte resembled the mucous cells in the dilution range in which they took up the dye.

The differences between these several metachromatic substances were further illustrated by the differential extractability of the dye.

Experiment 3. A differential rate of colour removal occurs when a stained preparation is heated with dilute H_2SO_4. Sections were stained in the cold

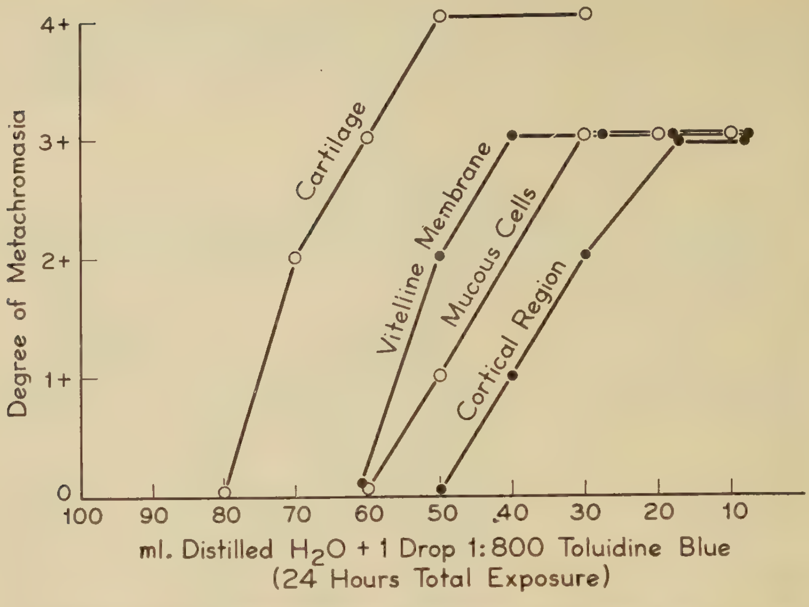

FIG. 1. Relative degrees of metachromasia as determined after staining with toluidine blue O, as a function of dye-dilution with a constant exposure-time. The vitelline membrane and oocyte cortex are compared with juvenile mouse-tail cartilage and mucous cells from frog small intestine.

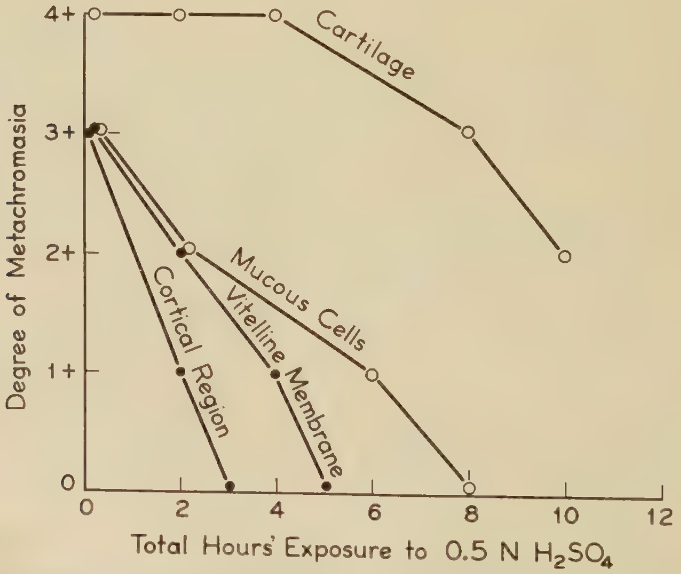

FIG. 2. Difference in metachromatic substances demonstrated by the time required for extraction of toluidine blue O by 0·5 N H_2SO_4.

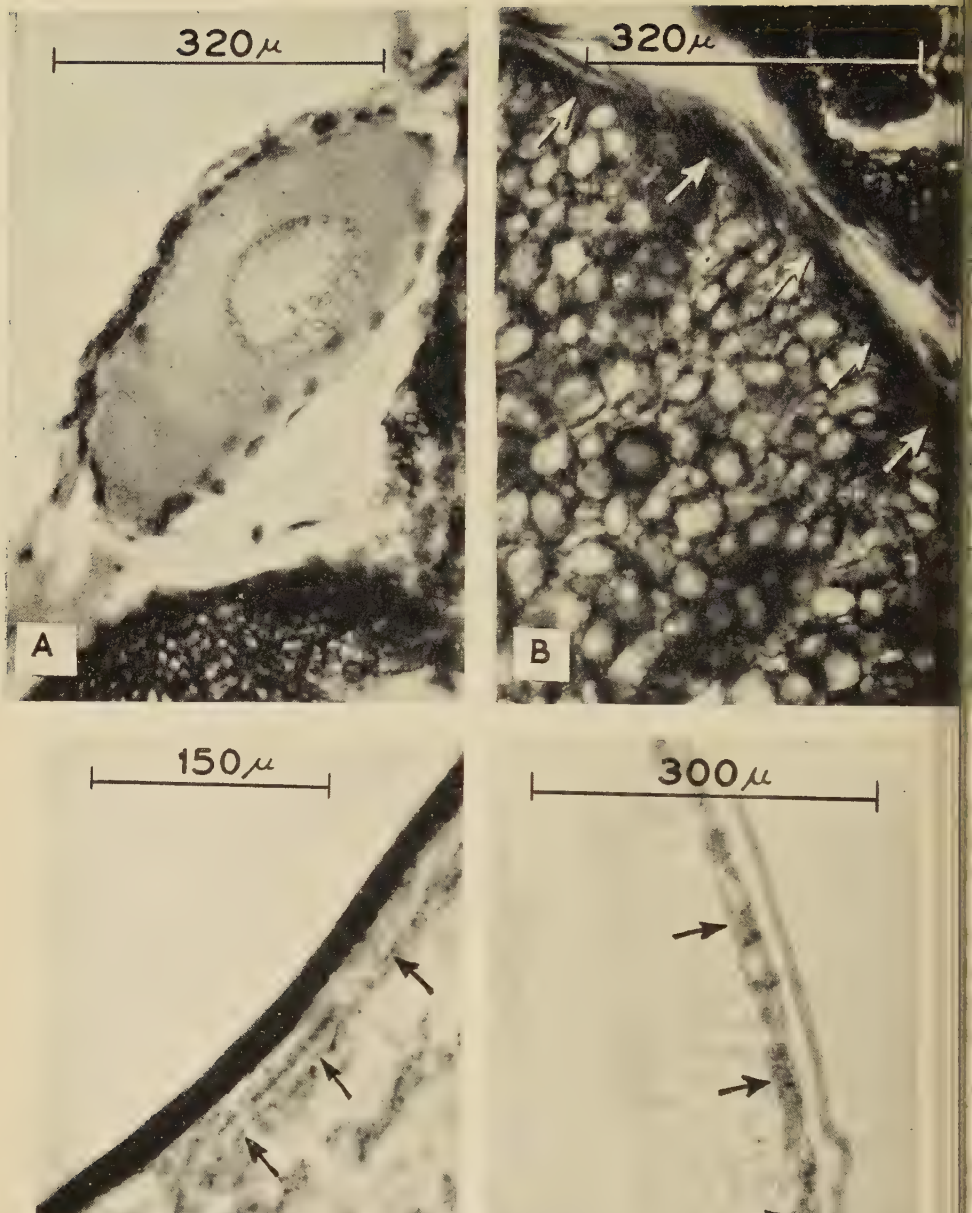

Fig. 3

R. M. ROSENBAUM

° C) for 12 h in a solution consisting of 1 drop of TBO (1:800) per ml of dis- led water. The resulting uptake of dye was evaluated in terms of an arbitrary lour standard. The sections were washed in cold tap-water for 3 to 5 h and en treated in 0·5 N H_2SO_4 at 56° C until the dye was extracted. The rate of sappearance of the dye was plotted as total time in acid against the intensity the dye bound at intervals of time, as shown in fig. 2. It is seen that cartilage ill stained with the initial intensity after 4 h in acid, while mucoitin or ucoitin-like complexes in small intestine and in the vitelline membrane and rtical region of frog oocytes were already partially or completely de- lourized.

In the three experiments described above, cortical metachromasia was found ly in oocytes undergoing active growth and yolk synthesis. Fig. 3, A shows stage I oocyte, the cortical region of which exhibited no metachromatic anules although the surrounding follicle cells were stained a deep blue. hile exhibiting no cortical metachromasia, however, oocytes at a pre-vitel- ne stage were found to contain granules in the cortex demonstrable by other ethods (Rosenbaum, 1957). Mature stage III oocytes showed an intensely etachromatic region just under the vitelline membrane (fig. 3, B); granules this region reacted in addition with a number of other reagents. It seems at the occurrence of cortical metachromasia is a character of mature oocytes though this region is already distinguishable in younger oocytes.

arbohydrate methods

After oxidation with periodic acid, most polysaccharides stain red violet with chsin-sulphite and yellow with 2,4-dinitrophenylhydrazine (DPH). A series experiments was undertaken to test the response of the cortical region of the cyte to various modifications of these two reactions.

Experiment 4. To eliminate glycogen, oocytes were fixed in warm aqueous xatives such as 10% formalin, Bouin's fluid, or 4% basic lead subacetate fol- wed by formalin. As an additional precaution, sections were exposed to liva for half an hour at 37° C. These sections were then stained by the andard periodic acid Schiff (PAS) technique.

The fully formed vitelline membrane stained intensely, the pellicles of the

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FIG. 3 (plate). A, stage I (previtelline) oocyte stained with 1% aqueous toluidine blue O ter fixation in basic lead subacetate and 10% formalin. Follicle cell nuclei are stained in- nsely blue. There is no evidence of cytoplasmic staining.

B, stage III (vitellogenic) mature oocyte stained after fixation as in A. There is a distinct etachromatic region under the follicle cells at the periphery of the oocyte (arrows). This gion is granular in appearance and extends between the most peripheral yolk platelets.

C, mature oocyte stained by the PAS method after formalin fixation and saliva digestion. AS positive granules (arrows) are seen beneath the darkly stained vitelline membrane and tween the most peripheral yolk platelets. Green filter.

D, mature oocyte stained with 2,4-dinitrophenylhydrazine (DPH) for 24 h followed by chsin-sulphite (PAS). The follicle cell layer, vitelline membrane, and ooplasmic ground bstance give a faint PAS-positive reaction. A DPH reaction (arrows) is seen in the cortical gion just under the vitelline membrane. Granules between the yolk platelets are pigment. d filter.

yolk platelets stained faintly. In addition, granules in the cortical region were intensely PAS-positive in stage II and III oocytes. In confirmation of other studies, it was shown that this region is one of considerable rigidity, since no shift in the localization of the material that was stainable by the PAS method was detected after centrifugation of the oocytes at 1,200 r.p.m. for as long as 60 min. Such treatment was more than adequate for the translocation of yolk platelets, glycogen granules, pigment, and the germinal vesicle.

Experiment 5. Paraffin sections of oocytes fixed in neutral formalin or lead subacetate were oxidized in periodic acid for 10 min, washed in distilled water, and then placed for varying periods in a mixture of 17 ml 30% ethanol saturated with DPH and 13 ml 0·2 N sodium acetate. After staining, the slides were rinsed thoroughly in several changes of 17% ethanol, dehydrated, cleared, and mounted.

The cortical granules around the entire periphery of the oocyte just under the ooplasmic membrane reacted positively. These granules were topographically identical with those observed to react positively with PAS and, in older oocytes, metachromatically with TBO (fig. 3, c).

With a constant oxidation time but with various periods of exposure to DPH some of the polysaccharides reacted more slowly with the phenylhydrazine reagent than did others. Thus, when slides were stained with DPH for short periods and then placed in fuchsin-sulphite, some granules were able to recolourize the reagent. However, sections exposed to DPH for 24 h were no longer capable of reacting with Schiff's reagent.

Experiment 6. Sections of oocytes that had been fixed in 4% basic lead subacetate or 10% neutral formalin were hydrated, then placed in periodic acid for 10 min at room temperature, after which they were washed thoroughly in distilled water and immersed in 70% ethanol for 15 min. They were then placed in DPH for 4 to 36 h at room temperature, rinsed in 17% ethanol and again placed in distilled water for 15 min. They were then exposed to fuchsin-sulphite for 45 min in the standard manner.

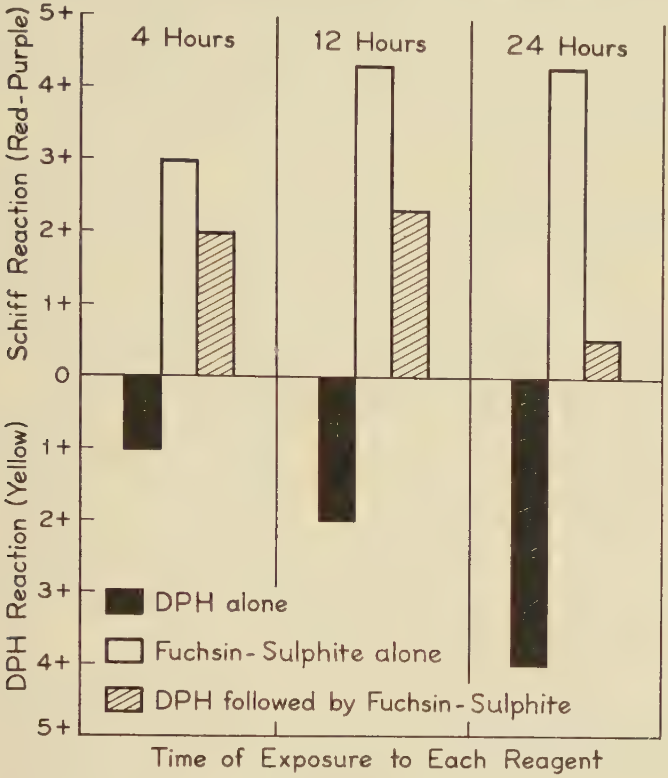
When a section was immersed in DPH for only 4 h and then placed in fuchsin-sulphite, the granules present were stained red. By 8 to 16 h some of them had bound DPH but many were still reactive with Schiff's reagent. By 24 h all the available polysaccharide material in the granules had combined with DPH and no red staining reaction was obtained on exposure to fuchsin-sulphite (figs. 3, D; 4).

Effects of proteolytic enzymes

The surface coat of the living frog egg and embryo has been shown to be especially susceptible to trypsin (Townes, 1953). It was thus of interest to relate the trypsin susceptibility of the living egg with a possible specific proteolytic action on the cortical region of the fixed oocyte.

Experiment 7. Crystallized undialysed trypsin (Armour) was dissolved in 0·0025 N HCl (pH 3·0) to make a 1% solution. This was dialysed against 0·0025 N HCl at 15° C overnight to obtain a salt-free preparation. Final solu-

ns were made up in M/15 phosphate buffer at pH 7·3–7·5 to give dilutions
1:1, 1:2, 1:4, and 1:8 of the stock 1% trypsin solution. Both fresh, unfixed
cytes and sections of oocytes fixed in basic lead subacetate followed by forma-
n were placed in the trypsin solutions for varying periods at 37° C. After



. 4. The results of separate and combined treatments with 2,4-dinitrophenylhydrazine
d fuchsin-sulphite on the cortical granules of stage III (mature) oocytes. At a given exposure
e there is a direct relationship between material coloured by DPH and that remaining
ctive to Schiff's reagent. This is seen by comparing the intensity of the reaction with
DPH alone and DPH followed by fuchsin-sulphite.

posure, the unfixed oocytes were fixed in 4% basic lead subacetate and
malin. They were then dehydrated, embedded in paraffin, and cut at 8 to
μ. Sections of oocyte material treated with the trypsin solutions before and
er fixation were then stained with TBO or PAS as previously described.

After trypsin digestion, the metachromatic, PAS-positive granules in the
tex of the maturing oocyte was no longer distinguishable (table 3). Upon
longed exposure to trypsin, the vitelline membrane and cortex proper dis-
egrated. Pretreatment of fixed oocyte material with thioglycollate had no
ect on the rate of trypsin digestion, and this suggests the absence of a
atin-like molecular arrangement (Goddard and Michaelis, 1934).

The point of attack by trypsin is primarily at the amide linkages of arginine and lysine residues and as such is different from that of chymotrypsin, which requires the presence of an aromatic ring containing tyrosine or phenylalanine residues. The effect of chymotrypsin was therefore investigated.

Experiment 8. Chymotrypsin (Armour, crystalline, salt-free) was prepared in M/15 phosphate buffer (pH 7·8–8·0) at 0·1%, 0·5%, 0·25%, and 0·01%. Sections of oocytes fixed in basic lead subacetate were placed in concentrations of the enzyme for varying lengths of time. Upon removal from these solutions, the sections were stained to find evidence of metachromasia.

TABLE 3

Effects of trypsin and chymotrypsin on fixed sections of stage III oocytes of R. pipiens

Enzyme	*pH of buffer*	*Final enzyme conc. (mg/100 ml buffer)*	*Vitelline membrane of stage III oocyte*	*Cortical granules in stage III oocyte*
Cryst. trypsin	7·3	500	*	*
		333	*	*
		200	Removed in 3 h	Removed in 1 h
		111	Removed in 5 h	Removed in 1 h
Cryst. chymotrypsin	7·5	100	*	*
		50	*	*
		25	Removed in 1 h	Removed in ½ h
		10	Removed in 1 h	Removed in ½ h

* Disintegration of entire oocyte.

Although it is difficult to compare the two enzyme preparations owing to different units of weight based on assay, it is seen from table 3 that the proteolytic effect of chymotrypsin appeared generally more severe than that obtained with trypsin. Both, however, appeared to attack the cortical region readily, whereas the central ooplasm was relatively refractory to digestion during the identical period of exposure.

Experiments with hyaluronidase from bull testis revealed that this preparation had no effect on the cortical region of the *R. pipiens* oocyte.

DISCUSSION

The present observations indicate that it is possible to characterize cytochemically the cortical region of the developing oocyte of *R. pipiens*. Throughout the study, relations of significance to cleavage and the development of the future embryo were kept in mind.

The presence of chromotropic material in the cortex of the oocyte allows a number of speculations regarding the nature and significance of this reaction in the present material. In a broad sense, metachromasia detects substances of high molecular weight with free anionic groups. These may include nucleic acids and anionic mucopolysaccharides; substances of high molecular weight ntaining ester sulphates are particularly strong chromotropes in the metaromatic reaction (Schubert and Hamerman, 1956).

As reviewed by Meyer (1938, 1945), the two principal sulphuric acid ester lysaccharides found in animal tissue consist of a hexosamine and glucuronic, etic, and sulphuric acids. One of these, chrondroitin sulphate, is an important component of cartilage, Wharton's jelly, and most other connective tissues. he other, mucoitin sulphate (Levene, 1925), is found chiefly in the intestinal ucosa. Hempelman (1940), in studying the distribution of these two subances in animal tissue, was able to differentiate them by methods similar to ose reported here. The similarity of the cortically distributed chromotropic aterial and mucoitin polysaccharides was revealed here by means of differtial uptake of toluidine blue O. Confirmation of the carbohydrate nature of e material was provided by positive reactions to fuchsin-sulphite and diniophenylhydrazine after periodic acid oxidation.

Heilbrunn and his co-workers (Heilbrunn and Wilson, 1950) have described e presence of sulphuric acid ester polysaccharides in the eggs of marine inrtebrates. These appeared to resemble heparin and to bear a relationship to anges in protoplasmic viscosity during cleavage. Heparin is considered to be mucoitin polysulphuric acid (Jorpes and Bergstrom, 1937). At the beginning this paper the importance of viscosity changes in the cortex of the frog egg uring cleavage and subsequent morphogenetic movements was noted. Kelly 954) has made a brief survey of metachromasia in various eggs but presents detailed data for amphibian material. One recent study (Wartenberg, 1956) as described a metachromatic reaction and PAS-positive granules in the rtex of the *Xenopus* oocyte. Similarly, polysaccharide material has been entified with the egg cortex of a stickleback, *Pungitus* (Kusa, 1953), and the edaka, *Oryzias* (Aketa, 1954).

From our present knowledge, it appears that the cortical granules consist hydrophilic protein-polysaccharide complexes. Monné and Slautterback 950) seem to be dealing with similar substances in the sea-urchin egg. These orkers state that they were able to identify three classes of mucopolysacarides in unfertilized and fertilized eggs. However, at the present time there some question as to the reliability of histochemical techniques for disnguishing so minutely between the complex molecules of high molecular eight that enter into the basic composition of the oocyte. In addition, our owledge of the basic structure of such polysaccharides in living systems akes a detailed consideration of them exceedingly difficult at the present ne.

The material in the cortex of the frog oocyte appears to be bound as a proin complex, the degree of polymerization of which is different from that of e interior ooplasm. It has been generally assumed that salts of heavy metals use precipitation of anionic polysaccharides and protein (Schubert and amerman, 1956). Formalin, as far as is known, reacts with proteins and may the polysaccharide to protein but does not react with anionic tissue polyccharides directly. With these facts in mind, it seems likely that formalin treatment after lead subacetate fixation causes a binding of polysaccharide to the protein ground-substance of the cortex. Other experiments (Rosenbaum, 1953) have shown that such a binding reaction may occur with alcohol dehydration after lead subacetate fixation. In fact, any method bringing about denaturation of protein without dissolving out the precipitated polysaccharide material will tend to cause such binding.

The cortical granules were found to be more sensitive to trypsin and chymotrypsin digestion than was the internal ooplasm. It is convenient to consider such differential activity in relation to the nature of the amino-acid residues in the two types of protoplasm. Whether or not the specific arrangement of amino-acid moieties in the cortical proteins governs their physical properties is, of course, still unknown. That the cortex is more rigid than the internal ooplasm has been demonstrated especially in invertebrate eggs, where cyclic changes in viscosity have been directly related to events associated with cleavage. In living material, the selective disruption of the cortex by trypsin has been demonstrated in the eggs of *Dendraster* (Moore, 1952) and of amphibia (Townes, 1953), where it results in severe abnormalities in later development. Recently, the membranes of living rat and rabbit eggs have been subjected to trypsin and chymotrypsin (Braden, 1952) with closely parallel results. Braden (1952) was unable to demonstrate any effect on the egg membranes of the rat and rabbit with hyaluronidase.

The recent visualization of granules in the cortex of *R. pipiens* oocytes by means of the electron microscope (Kemp, 1956) is especially interesting. It appears that the granular material originates in the peripheral ground cytoplasm. It is possible that the granules revealed under the electron microscope are identical or closely related to the metachromatic, Schiff-positive material demonstrated here. In this respect, it is interesting to compare electron micrographs of the metachromatic granules in the mast cell (Smith and Lewis, 1957) with the cortical granules of the frog oocyte (Kemp, 1956). Under fairly similar conditions of osmium fixation, the two sets of granules appear remarkably similar in their relationship to each other, their internal structure, and the degree of electron density.

Some of the preliminary experiments of this series comprised a portion of a thesis submitted to the Graduate School of Arts and Science, Department of Biology, Harvard University. I express my thanks to Professor Leigh Hoadley for his encouragement during the early part of this work. I also express my thanks to Professor Alfred Angrist and Dr. Helen Wendler Deane for their critical reading of the manuscript.

REFERENCES

AKETA, M., 1954. Embryologica, **2**, 63.
ALLEN, R. D., and HAGSTROM, B. E., 1955. Exp. Cell Res., suppl., **3**, 1.
BRADEN, A. W. H., 1952. Austr. J. Sci. Res. B, **2**, 460.
DALCQ, A., 1940. Arch. Biol., **51**, 387.

ODDARD, D. R., and MICHAELIS, L., 1934. J. biol. Chem., **106**, 605.
EILBRUNN, L. V., and WILSON, W. L., 1950. Protoplasma, **39**, 389.
EMPELMAN, L. H., 1940. Anat. Rec., **78**, 197.
OLTFRETER, J., 1943. J. exp. Zool., **93**, 251.
—— 1948. Ann N.Y. Acad. Sci., **49**, 684.
ORPES, E., and BERGSTROM, S., 1937. J. biol. Chem., **118**, 447.
ELLY, J. W., 1954. Protoplasma, **43**, 25.
EMP, N. E., 1956. J. biophys. and biochem. Cyt., **2**, 281.
USA, M., 1953. Annot. Zool. Jap., **26**, 138.
EVENE, P. A., 1925. *Hexosamines and mucoproteins*, London (Longmans).
EYER, K., 1938. Symp. Quant. Biol., **6**, 91.
—— 1945. Adv. in Protein Chem., **2**, 249.
ONNÉ, L., and SLAUTTERBACK, D. B., 1950. Exp. Cell Res., **1**, 477.
OORE, A. R., 1952. Ibid., **2**, 264.
OSENBAUM, R. M., 1953. Ph.D. thesis, Harvard Univ.
—— 1957. Anat. Rec., **127**, 359.
ELMAN, G. G., and WADDINGTON, C. H., 1955. J. exp. Biol., **32**, 700.
CHECTMAN, A. M., 1937. Science, **85**, 222.
CHUBERT, M., and HAMERMAN, D., 1956. J. Histochem. Cytochem., **4**, 158.
MITH, D. E., and LEWIS, Y. S., 1957. J. biophys. and biochem. Cyt., **3**, 9.
OWNES, P. L., 1953. Exp. Cell Res., **4**, 96.
ARTENBERG, H., 1956. Acta Histochem., **3**, 25.

Activity of the Corpora Allata during Pupal Diapause in *Mimas tiliae* (Lepidoptera)

By K. C. HIGHNAM

(*From the Department of Zoology, the University of Sheffield*)

With one plate (fig. 2)

SUMMARY

Histological observation indicates that the corpora allata of *Mimas tiliae* are secretory uring pupal diapause, but become inactive by the end of the low-temperature period vhich terminates diapause.

Removal of the corpora allata, together with the corpora cardiaca, from the diaausing pupa increases the thickness of the hypodermis and decreases the number of at-body inclusions (compared with operated controls), but does not result in any isible signs of diapause break.

The oxygen uptake of the pupa increases by about 60% by the end of the lowemperature period, compared with the diapausing pupa.

It is probable that the corpora allata play some part in the maintenance of diapause, ossibly by exercising some control over the fat-body metabolism. This role is subervient to that of the neurosecretory cells in the brain, together with their associated orpora cardiaca.

INTRODUCTION

IN a previous paper on the problem of diapause 'break' in the pupa of *Mimas tiliae* (Highnam, 1958) it was shown that the cerebral neurosecretory ells, together with their associated corpora cardiaca, play an important part n terminating diapause in this insect. The mechanism of this process, in vhich the prothoracic glands are also involved (Williams, 1947), is essentially imilar to that controlling moulting and metamorphosis in most insects Wigglesworth, 1955).

The present paper is concerned with demonstrating that variations in the ecretory activity of the corpora allata occur in the pupal stage of *M. tiliae*, vhich indicate that the glands are involved in the continuation of diapause nd in the process of diapause development at low temperatures that brings liapause to an end.

MATERIAL AND METHODS

Samples were taken of *M. tiliae* in diapause, of pupae after 1, 2, 3, and 4 veeks or more at the temperature of 3° C which terminates diapause (Higham, 1958), and of post-diapausing pupae until adult emergence. These were xed in Bouin, Duboscq-Brasil, Susa, or Zenker, embedded in paraffin wax fter stripping off the pupal cuticle, and sectioned at 6 μ. Serial sections were tained with Weigert's haematoxylin and eosin, or with Mallory's or Masson's echnique. In those series in which measurements of gland or cell-volumes vere made, care was taken that in each the material spent an exactly similar

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time in the fixative (Bouin), and was embedded in paraffin wax at the same temperature for the same time.

Glands were extirpated by a technique similar to that described by Williams (1947). The method is outlined on p. 176.

Oxygen uptakes of chopped pupal tissues were measured in the Warburg respirometer according to directions given in Dixon (1951).

RESULTS

The histology of the corpora allata during diapause

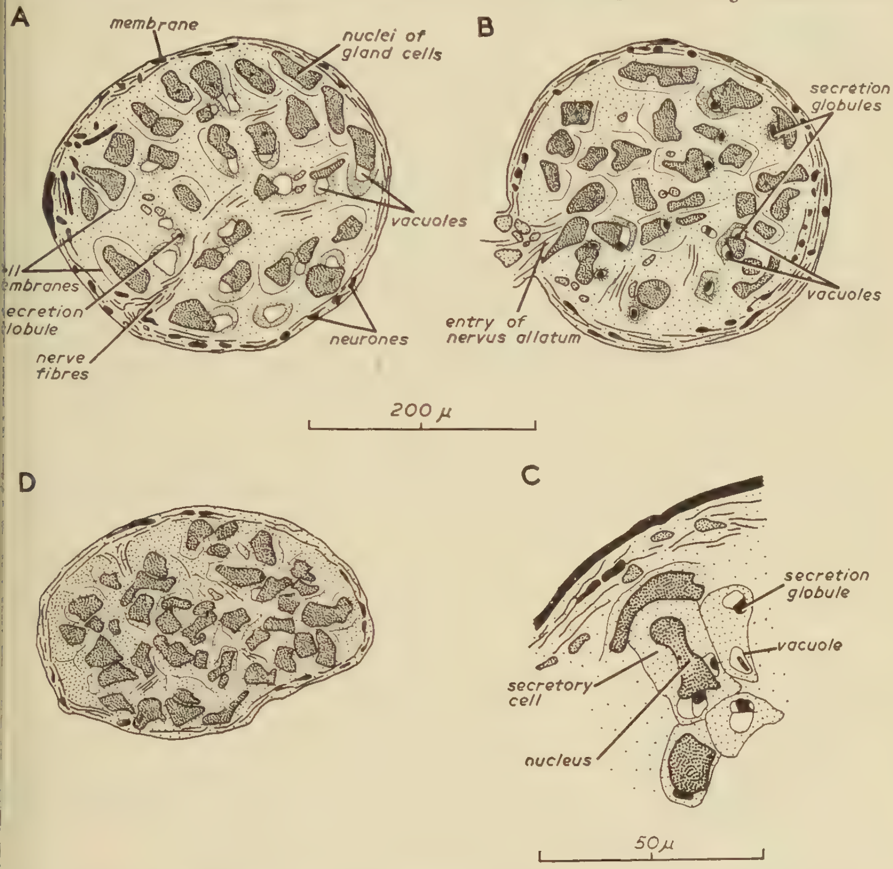
The general relationship of the corpora allata with the rest of the endocrine system has already been described (Highnam, 1958). Each corpus allatum is surrounded by a very obvious connective tissue coat, about 5 μ in thickness (fig. 1). The membrane stains blue or green with Mallory's or Masson's technique. The thick membrane must be added at pupation since the larval corpus allatum is surrounded by a membrane which is very thin. It is probably formed by haemocytes (Wigglesworth, personal communication). Immediately beneath the membrane are a number of neurones with axons running parallel with the surface of the gland. From certain points on the periphery, nerve-fibres pass to the inside (fig. 1, A) and many neurones are present at these points. Besides these small nerves, a large nerve (the nervus allatum) enters each gland from the corpus cardiacum of the same side. The nervus allatum is the point of origin of the fibres running beneath the membrane. Some of the fibres form a very fine nerve which leaves the posterior face of each corpus allatum and runs in the direction of the aorta.

The secretory cells which make up the bulk of each corpus allatum are large and irregularly shaped (fig. 1, A). The cell-membranes are often difficult to detect. The cytoplasm has a fibrous appearance with the fixatives used, and is weakly acidophil. Large numbers of vacuoles are present throughout the glands and contain very acidophil globules or granules (fig. 1, A), which seem to be secretory material elaborated by the cells, since in some cases acidophil bodies are found in the cytoplasm surrounding the vacuoles. This surrounding cytoplasm differs from that of the rest of the cell in its more homogeneous appearance, and greater acidophilia. The nuclei of the secretory cells are large, with an irregular shape (fig. 1, A). They are densely granular, with a single well-defined nucleolus. The nuclei are arranged more or less radially around the periphery of the glands, but are oriented at random in the centres.

The corpora allata are richly supplied with tracheae. A large branch springs from each of the upper two tracheal trunks which enter the head from the prothorax and passes to the corpus allatum of the same side. As the branch enters the gland, it breaks up into a number of smaller branches, the nuclei of the lining epithelium of which can be seen in prepared sections.

Histology of the corpora allata during diapause development at 3° C

During the latter part of the low-temperature treatment, the histological appearance of the corpora allata undergoes a very marked change. For the first weeks at 3° C, the glands are large (mean diameter 130 to 150 μ), with large clei separated by wide tracts of cytoplasm (fig. 1, B). The cytoplasm is cuolated, and the vacuoles often contain acidophil inclusions (fig. 1, C). In neral appearance, therefore, the corpora allata during the first 3 weeks at the



G. 1. Camera lucida drawings of sections of corpora allata. A, in the diapausing pupa. ote the size of the gland, the distance apart of the nuclei, and the presence of vacuoles, some ith inclusions. B, after 1 week at 3° C; features similar to A. C, detailed drawing of part of B show the intracellular vacuoles and inclusions. D, after 4 weeks at 3 C. The gland is small, ith few vacuoles and no inclusions. The nuclei are small and aggregated closely together.

w temperature are very little different from the glands of the unchilled iapausing pupa (fig. 1, A).

By the end of the fourth week at 3° C, the glands are considerably smaller nean diameter about 100 μ) with smaller nuclei aggregated more closely gether (fig. 1, D). Vacuoles are present in the cytoplasm but these are not surunded by the differentiated cytoplasm seen in the diapausing and early low-mperature glands. There are no inclusions in the corpora allata at this time.

No mitoses occur in the corpora allata during the diapause and low-temerature periods. It is possible, therefore, to compare directly the size of the glands during diapause and after 1, 2, 3, and 4 weeks or more at 3° C: either by measuring the volume of the glands, or their cell-volume, at these different times. As will be shown later, these are methods for determining objectively the secretory activity of the corpora allata.

Volume of the corpora allata

The volume of each corpus allatum was calculated from the mean section area. Assuming that the corpora allata are spherical,

$$\text{volume} = (\text{mean section area})^{3/2} \times 2\sqrt{\tfrac{3}{2}}\pi.$$

The mean section area was calculated by drawing every second section of a corpus allatum under a camera lucida, and measuring the areas of the drawn sections with an 'Allbrit' precision disk planimeter. The magnification being known, the values for the actual gland sections in square microns are easily obtained. The sum of the section areas divided by the number of sections drawn gives the mean section area. The gland-volumes can then be calculated. Table 1 gives the values obtained for diapausing and low-temperature pupae.

TABLE 1

Volumes of corpora allata during diapause and diapause development

Series	*Mean volume of corpora allata* ($\times 10^{-6}\ \mu^3$)	*Standard error*	*Comparison of means*
a_0	1·34	±0·09	$P > 0{\cdot}1$
a_1	1·22	±0·20	$P > 0{\cdot}1$
a_2	1·81	±0·22	$P > 0{\cdot}1$
a_3	1·93	±0·33	$P << 0{\cdot}01$
a_4	0·75	±0·05	$P < 0{\cdot}1 > 0{\cdot}05$
a_5	0·56	±0·05	$P > 0{\cdot}1$
a_6	0·62	±0·05	

a_0 Represents a sample of pupae diapausing at 25° C. a_1, a_2, ..., a_6 represent samples taken 1, 2, ..., 6 weeks after transfer to 3° C. In each sample the volume is the mean of 10 corpora allata from 5 individuals. Means compared by the t-test for small samples.

The difference between the volumes of corpora allata after 3 and 4 weeks respectively at 3° C is well marked and is statistically significantly different (table 1). The gland-volumes in the sequences a_0 to a_3 (diapausing and for the first 3 weeks of the low-temperature period) and a_4 to a_6 (after 4 to 6 weeks at the low temperature) are not significantly different from one another (table 1).

The large standard errors of the means of the volumes of corpora allata for each sample taken during the diapause period and for the first 3 weeks at the low temperature indicate a large variation in volume size between individuals, which may be obscuring differences in mean volume between samples. This variation may be obviated by estimating the cell-volume of the corpora allata (compare Brisson, 1949). If the corpora allata are larger in one individual than in another, after the same treatment, this must be due to the glands consisting of a greater number of cells. Any variation in cell-volume between amples is then likely to be absolute, and not dependent upon individual ariation.

ell-volume of corpora allata

Four or five central sections were drawn under a camera lucida and the areas f the drawn sections measured with an 'Allbrit' precision disk planimeter. he number of gland cell nuclei were counted in each drawn section, and the ell-volume calculated from the formula

$$V = a \times t/n,$$

vhere V is the cell volume, a the area of the section in square microns, t the ection thickness in microns, and n the number of nuclei in that section. The ean of the cell-volume was found for each individual.

It should be stressed that all gland nuclei were counted in each section rawn. Such a count included parts of nuclei divided when the section was ut, as well as whole nuclei. Since the number of nuclei divided depends upon he nuclear size and the section thickness (Abercrombie, 1946), a true nuclear ount cannot be obtained by this method. But methods recommended for bviating this difficulty are unnecessary when comparative values are required.

The results obtained are shown in table 2. As with the volume estimations, he figures show a marked decrease in cell-volume between the third and ourth weeks at 3° C.

TABLE 2

Cell-volumes of corpora allata during diapause and diapause break

Series	*Mean cell-volume* ($\times 10^{-3}\ \mu^3$)	*Standard error*	*Comparison of mean*
a_0	3·15	±0·13	$P > 0{\cdot}1$
a_1	3·10	±0·17	$P > 0{\cdot}05 < 0{\cdot}1$
a_2	3·51	±0·14	$P > 0{\cdot}1$
a_3	3·21	±0·12	$P << 0{\cdot}01$
a_4	1·60	±0·06	$P < 0{\cdot}01$
a_5	1·21	±0·04	$P > 0{\cdot}1$
a_6	1·45	±0·05	

Symbols as in Table 1. Means compared by the t-test for small samples.

By comparison with the corpora allata of other insects during the times that hey are known experimentally to be producing hormone (Wigglesworth, 934; Burtt, 1937; Mendes, 1948), it would seem that the large corpora allata vith widely spaced nuclei are actively secreting, while the smaller glands are nactive. Mendes (1948) has made a detailed examination of the corpora allata f *Melanoplus differentialis*, the experimental determination of whose function ad been previously resolved (Weed, 1936; Pfeiffer, 1939, 1945). Mendes tates: 'During the inactive phases they [the secretory cells of the corpora llata] are reduced in size. . . . During the active phase they increase in cytoplasmic and nuclear volume.' Wigglesworth (1934) gives drawings of sections of the corpus allatum of *Rhodnius* showing comparable changes during the experimentally determined periods of activity and inactivity of the gland. There is little doubt, therefore, that the corpora allata of the pupa of *Mimas tiliae* are secretory during the diapause period and for the first 3 weeks at 3° C, and that they are nonsecretory thereafter.

Removal of the corpora allata and cardiaca

It can be demonstrated by removal of the glands that the secretion of the corpora allata has some physiological effect on the diapausing pupa. Owing to the intimate association of the corpora allata with the corpora cardiaca, it was found necessary to remove both pairs of glands, but it is unlikely that the corpora cardiaca are responsible for the effects to be described, since these glands show no histological signs of secretory activity in the diapausing pupa (Highnam, 1958).

The corpora allata and cardiaca were removed by a modification of the method described by Williams (1947). The head region of the diapausing pupa was severed by an annular cut, the fore-gut ligatured with a silk thread, cut through in front of the ligature, and folded back into the body. The head region was then removed, the corpora allata and cardiaca separated from the brain and the latter transferred back into the body of the pupa. The cut end of the pupa was closed with a perforated perspex circle, sealed to the animal with paraffin wax. A few crystals of phenylthiourea were inserted through the perforation to prevent the browning and eventual death of the operated pupae due to the enhanced activity of the tyrosine-tyrosinase system after wounding (Williams, 1947). Any air in the pupa was displaced with physiological saline and the perforation closed with a blob of wax. This method ensures that no trace of the corpora allata remains in the operated pupae. Seven out of 12 operated pupae, and 3 out of 5 controls (which were treated similarly except that the corpora allata and cardiaca were replaced with the brain) survived for more than 15 days at 25° C. No adults emerged from either the experimental or the control series during the time at 25° C, and dissection revealed no visible adult development. Histological examination shows that the hypodermis has increased in thickness in the experimental pupae, compared with the controls (fig. 2, A, B), and their fat-bodies contain a much smaller number of inclusions compared with the controls (fig. 2, C, D), resembling, except for

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FIG. 2 (plate). Photomicrographs of prepared sections (fixed in Bouin, stained Masson's trichome) of hypodermis and fat-body. A, section of hypodermis and cuticle in which the endocrine system had been removed and replaced 15 days before (control for B).

B, hypodermis and cuticle of an individual from which the corpora allata and cardiaca had been removed 15 days before. Note the thickness of the hypodermis compared with A.

C, fat-body of pupa after the same treatment as A.

D, fat-body of pupa after the same treatment as B. Note the absence of inclusions compared with C.

E, fat-body of an unoperated pupa, after 12 days at 25° C after 4 weeks at 3° C. Inclusions absent, as in D.

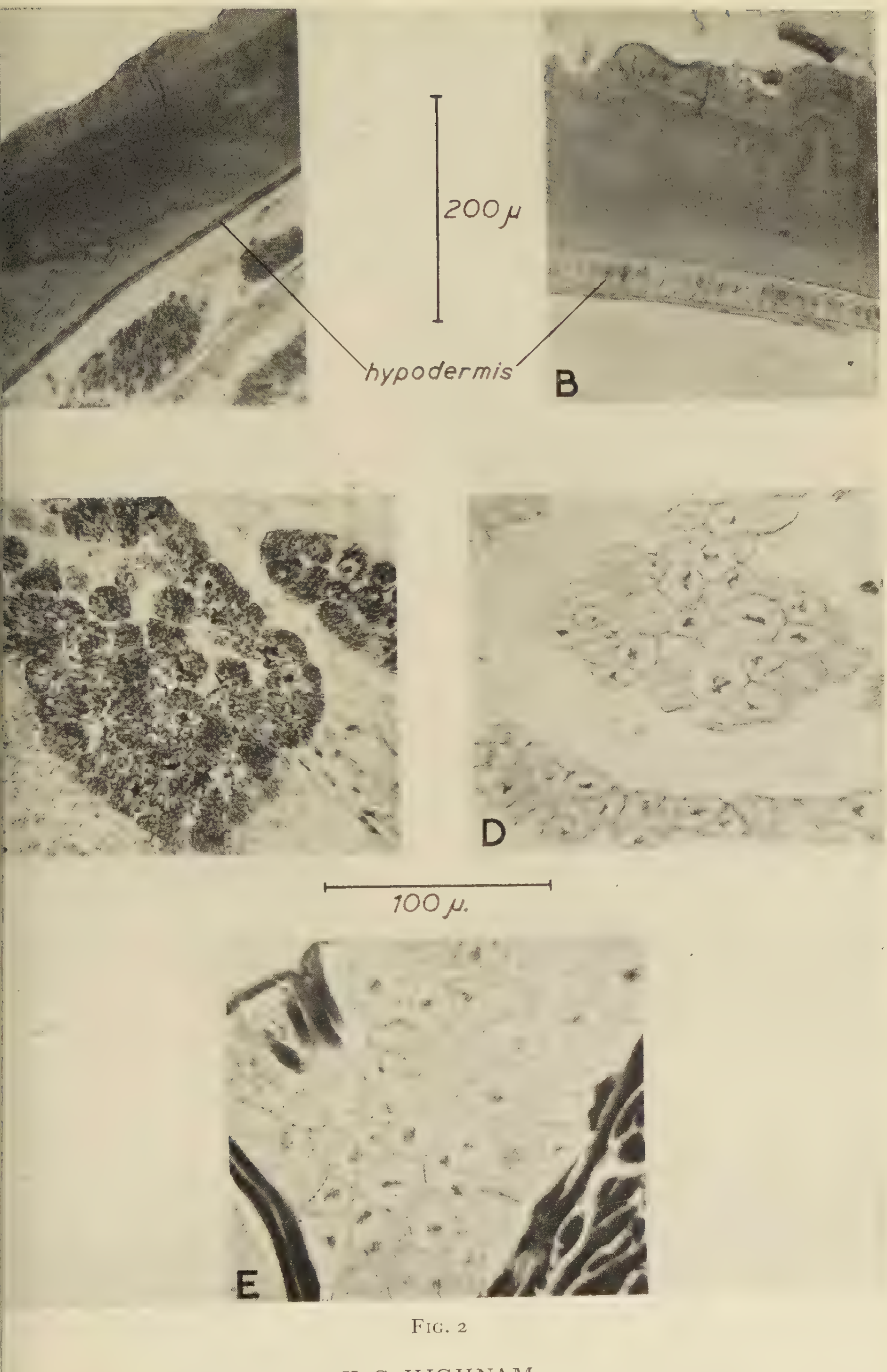


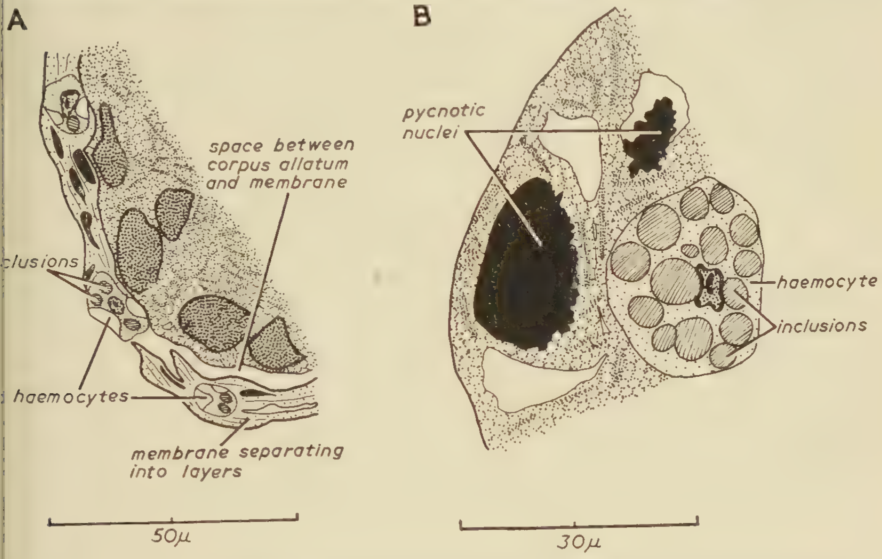
Fig. 2

K. C. HIGHNAM

e smaller size of the cells, the fat-body of a normal unoperated pupa a few ys before adult emergence (fig. 2, E).

stology of the corpora allata during post-diapause development at 25° C

During the time at 25° C, following at least 4 weeks at 3° C, the corpora ata show no sign of any secretory activity. The glands are phagocytosed and organized in a manner similar to many non-endocrine organs in the body.


A
space between corpus allatum and membrane
clusions
haemocytes
membrane separating into layers
50μ
B
pycnotic nuclei
haemocyte
inclusions
30μ

G. 3. Camera lucida drawings of the corpora allata during the time at 25° C after at least veeks at 3° C. A, after 2 days at 25° C. B, after 6 days at 25° C. The figures show stages in phagocytosis of the gland.

he membranes first separate from the glands and are phagocytosed by blood- lls (fig. 3, A). Then the glands proper are invaded by haemocytes. The gland iclei become pycnotic and shrunken (fig. 3, B), and many disappear. By the id of the tenth day at 25° C, the corpora allata have attained the adult form, ry similar to that of the larva in that the surrounding membrane is once ore thin. No mitoses have been observed in the corpora allata during this me, and it must be assumed that the adult glands are developed from some irt of the pupal glands after phagocytosis. The adult corpora allata are very uch smaller than the pupal glands.

upal oxygen uptakes

The present observations on the histology of the corpora allata suggest that ie glands are inactive by the end of the fourth week at 3° C. It has already een shown (Highnam, 1958) that the cerebral neurosecretory cells are in- ctive at this time, and that the non-endocrine organs of the body commence evelopment immediately the pupa is transferred to 25° C, after at least weeks at 3° C. It is concluded that the conditions for further development are established by the end of the low-temperature period, but visible development cannot occur because the temperature is too low.

Further evidence for this view has been obtained by measuring the oxygen uptake of chopped pupal tissues in a Warburg respirometer at different times during diapause and diapause development. The result (table 3) shows that there is no significant difference between the oxygen uptake of the diapausing pupa, and of the pupa after 1 or 2 weeks at 3° C. But after 4 weeks at this temperature, the oxygen uptake has increased by about 60%. No mean value for the oxygen uptake of the pupa after 3 weeks at 3° C is given in table 3. Since the uptake varied from 0·63 (3 pupae) to 1·46 cu. mm. O_2/mg dry weight/hour (2 pupae), a mean value has little significance.

TABLE 3

Pupal oxygen uptakes (cu. mm. O_2/mg dry weight/hour)

Sample	*No. of specimens*	*Mean O_2 uptake*	*Standard error*
Diapausing pupa . .	15	0·89	±0·09
After 1 week at 3° C .	5	0·91	±0·11
After 2 weeks at 3° C .	5	0·91	±0·06
After 4 weeks at 3° C .	10	1·48	±0·04
After 4 weeks at 3° C+ 6 days at 25° C .	4	2·30	±0·15
After 4 weeks at 3° C+ 12 days at 25° C .	3	2·47	. .

Uptakes determined at 35° C to give measurable values for the diapausing pupae.

Since the end of diapause is characterized by an increased oxygen uptake (Squire, 1936; Schneidermann and Williams, 1953), the present results indicate that the 'physiological diapause' which must underlie the morphogenetic one is broken before the end of the low-temperature period.

The increasing pupal oxygen uptakes during the post-diapause period follows the second half of the U-shaped curve so typical of respiration during normal pupation.

DISCUSSION

The histological evidence indicates that the corpora allata are the only endocrine organs actively secreting during diapause and that this activity ceases between the third and fourth weeks at 3° C. Regression of the glands (during low-temperature treatment) is probably brought about by the increasing neurosecretory activity of the brain at this time. Scharrer (1952*a*, 1952*b*) has shown in *Leucophaea* that unilateral section of the nervi cardiaci prevents the passage of neurosecretory material along the cut nerve and that this is accompanied by enlargement of the corpus allatum on the operated side. This would seem to imply that regression of the corpora allata is brought about by the flow of material from the cerebral neurosecretory cells. It is evident, however, from other experiments (Highnam, 1958), that the brain can override the physiological action of the corpora allata. Implantation of chilled brains and corpora rdiaca into diapausing pupae, where the corpora allata are active, terminates iapause. Regression of the corpora allata is thus incidental but not apparently sential to diapause break.

Extirpation of the corpora allata (and corpora cardiaca) from diapausing upae results in changes in the hypodermis and fat-body comparable with ose found in normal pupae in which diapause break has occurred. This early shows that the corpora allata are physiologically active during diapause d suggests that they are concerned with maintenance of diapause in *M. tiliae.* his conclusion is in agreement with the results of previous workers. Accord- g to Wigglesworth (1955) the corpora allata in the nymphs or larvae of all sects so far examined in some way prevent the development and differentia- on of adult characters. More recently, Williams (1956) has shown that the jection of corpus allatum extract into pupae '. . . causes a second pupal instar y opposing the pupal-adult transformation'. He calls the corpus allatum ctor the *status quo* hormone. In his previous work Williams (1946, 1947, 948, 1949) had concluded that diapause was merely induced by and con- nued in the absence of the brain and prothoracic gland factors known to be ecessary for diapause break. His recent (1956) results, coupled with those of e present investigation, suggest a more positive role for the corpora allata— e maintenance of diapause. In the diapausing pupa of *M. tiliae* the corpora lata are secretory whilst the neurosecretory tissue in the brain is almost in- ctive. During diapause development, increasing amounts of the brain factor e produced (Highnam, 1958). It seems probable that the transition from dia- use to diapause development depends on a change in the balance between *atus quo* and 'developmental' factors, and that when sufficient of the latter as been produced, the prothoracic glands are activated and diapause break ccurs. Other evidence in favour of a corpus allatum factor during diapause afforded by the synchronous diapause of insect parasites with their insect osts (Salt, 1941). This phenomenon is difficult to explain by any theory f diapause which involves merely the absence of developmental factors.

Evidence regarding the action of the corpus allatum factor is conflicting. horacic muscles from allatectomized *Leucophaea maderae* show an increased xygen uptake *in vitro*, in comparison with normal insects (Samuels, 1956), d the experiments of L'Hélias (1953) on *Dixippus morosus* suggest that this associated with an increase in the carbohydrate content of the tissue. The creased oxygen uptake at the end of diapause in *M. tiliae* may have a similar xplanation. The decreased activity of the corpora allata in the late pupa is ccompanied by a reduction of inclusions in the fat-body, which signifies some hange in its metabolism.

Contrary results have been obtained with adult *Calliphora erythrocephala* d adult *Periplaneta americana*. In the former, allatectomy lowers the oxygen ptake by 20% (Thomsen, 1949), and in the latter it causes an increased de- osition of fat in the fat-body (Bodenstein, 1953).

The results of the present work favour the suggestion of Andrewartha (1952) at the 'intractability' of food reserves governs the onset of diapause. The inability of the diapausing pupa to utilize its food reserves is due possibly to a hormonal control over the fat-body exercised by the corpora allata.

I am grateful to Professor L. E. S. Eastham, in whose department this work was carried out, for his continued interest and encouragement, and to Dr. F. J. Ebling for many interesting discussions on the measurements of organ volumes from prepared slides. My best thanks are due to Dr. F. Segrove for his helpful criticism of the original manuscript.

REFERENCES

Abercrombie, M., 1946. Anat. Rec., **94**, 238.
Andrewartha, H. G., 1952. Biol. Rev., **27**, 50.
Bodenstein, D., 1953. J. exp. Zool., **124**, 105.
Brisson, C. J., 1949. Bull. Biol., Suppl., **34**, 1.
Burtt, E. T., 1937. Proc. Roy. Soc. B, **124**, 13.
Dixon, M., 1951. *Manometric methods.* Cambridge (University Press).
L'Hélias, C., 1953. C.r. Acad. Sci. Paris, **236**, 2164.
Highnam, K. C., 1958. Quart. J. micr. Sci. (in press).
Mendes, M. V., 1948. Biol. Bull. Wood's Hole, **94**, 194.
Pfeiffer, I. W., 1939. J. exp. Zool., **82**, 439.
—— 1945. Ibid., **99**, 183.
Salt, G., 1941. Biol. Rev., **16**, 239.
Samuels, A., 1956. Biol. Bull. Wood's Hole, **110**, 179.
Scharrer, B., 1952*a*. Anat. Rec., **112**, 386.
—— 1952*b*. Biol. Bull. Wood's Hole, **102**, 261.
Schneidermann, H. A., and Williams, C. M., 1953. Ibid., **105**, 320.
Squire, F. A., 1936. Bull. ent. Res., **27**, 381.
Thomsen, E., 1949. J. exp. Biol., **26**, 137.
Weed, I. G., 1936. Proc. Soc. exp. Biol., **34**, 885.
Wigglesworth, V. B., 1934. Quart. J. micr., Sci. **77**, 191.
—— 1955. *Physiology of insect metamorphosis.* Cambridge (University Press).
Williams, C. M., 1946. Biol. Bull. Wood's Hole, **90**, 234.
—— 1947. Ibid., **93**, 90.
—— 1948. Ibid., **94**, 60.
—— 1949. Bull. Biol., Suppl., **33**, 52.
—— 1956. Proc. Roy. ent. Soc. Lond., C, **21**, 20.

Observations on the Mantle of the Lamellibranchia

By G. E. BEEDHAM

(*From the Department of Zoology, University of Glasgow*)

With two plates (figs. 3 and 4)

SUMMARY

The variation in histology between the epithelial regions of the mantle in *Anodonta* *gnea* concerned with the secretion of the different layers of the shell has been vestigated. The epithelia on the outer surface of the outer mantle fold which secrete e outer layer of the ligament and the corresponding outer calcareous layer of the lves are basically alike in structure. They are also comparable in appearance with e epithelium on the inner surface of the outer mantle fold which secretes the perioracum and with that of the fused outer folds responsible for the formation of the sion layer of the ligament. All these epithelial regions can readily be distinguished stologically from the epithelia of the general outer mantle surface and of the mantle thmus which produce the inner layers of the valves and ligament respectively.

The histological differentiation of the mantle epithelium in *Anodonta* is compared ith that of *Mytilus edulis* and *Ostrea edulis*. The degree of zonation of the epithelium n be correlated with the extent to which the shell layers differ in composition from e another. It is suggested that the variation in histological appearance between ithelia from different areas of the mantle is partly determined by the relative amounts non-calcareous material (conchiolin) which these epithelia produce and by the rate their secretory activity.

The different epithelial regions of the mantle in *Anodonta* and other lamellibranchs ry appreciably in their reactions to histochemical tests for alkaline phosphatase, bonucleic acids, and glycogen. In *Anodonta*, the epithelial cells of the outer mantle ld differ markedly from those which secrete the inner layers of the shell in that they hibit high alkaline phosphatase activity and appear to be rich in cytoplasmic bonucleic acids. In contrast, the epithelium of the general outer surface of the antle is distinguished by a high content of glycogen, a condition which also occurs many other bivalves. It is interesting to note that alkaline phosphatase can be monstrated in epithelia which secrete non-calcareous regions of the shell. In all the mellibranchs investigated, ribonucleic acids can be detected in relatively higher ncentration in epithelial cells concerned principally with the secretion of conchiolin.

INTRODUCTION

RECENT observations by Owen, Trueman, and Yonge (1953) have clarified the nature of the relationship between the mantle and shell in the amellibranchia. They pointed out that the valves and ligament, which gether constitute the shell, have fundamentally the same structure, each nsisting of a superficial periostracum and underlying outer and inner layers creted by different areas of the mantle. The periostracum and the outer yers of the valves and ligament are secreted respectively by the inner and ter surfaces of the outer mantle fold (Yonge, 1957), which bounds the tire mantle including the two ends of the narrow neck of tissue—the mantle thmus—situated beneath the ligament. The inner layer of the ligament is creted by the epithelium of the mantle isthmus which is continuous with

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that of the general outer surface of the mantle responsible for the production of the inner layer of the valves. The outer and inner layers of the valves and ligament are to be regarded, therefore, as modifications of the same two layers of the shell, since they are secreted by corresponding regions of the mantle.

These observations pose a number of interesting problems, which were investigated on *Anodonta cygnea* and other species of lamellibranchs. In the present paper, the variation in histological structure between the epithelial cells concerned with the secretion of the different layers of the shell of *A. cygnea* is described and is compared with that which occurs in *Mytilus edulis* and *Ostrea edulis*. Although differences in structure have been observed in species of *Anodonta* between the epithelial regions which secrete the periostracum and the outer and inner layers of the valves (de Villepoix, 1892; Rassbach, 1912; Siebert, 1913), a detailed comparison has not been made between the various epithelial zones underlying both the valves and the ligament. A study is also made of the histochemical properties of the different epithelial regions of the mantle in *Anodonta* and other lamellibranchs and the possible significance of these results in relation to aspects of shell secretion is discussed. Much work has been carried out on the histochemistry of the mantle (e.g. Bevelander and Benzer, 1948; Love and Frommhagen, 1953; Kado, 1953, 1954), but these investigations have been restricted to the mantle tissues concerned with the secretion of the valves.

The observations described in the present paper were made on material that had been fixed in Bouin's fluid, mercuric-formaldehyde, or other routine fixatives and embedded in paraffin wax or ester wax (Steedman, 1947). Descriptions of the histological structure of the epithelial cells are based on sections stained with Heidenhain's iron haematoxylin, Delafield's haematoxylin and eosin, and Mallory's and Masson's triple stains. Details of the histochemical tests used are given in the text.

THE HISTOLOGY OF THE MANTLE EPITHELIUM

A. cygnea

As in all lamellibranchs, the mantle edge of *Anodonta* is subdivided longitudinally into an outer secretory fold, a middle sensory fold, and an inner muscular fold (Siebert, 1913; Yonge, 1948) (fig. 1, A). The groove in which the periostracum is inserted, which lies between the outer and middle folds, is lined by two epithelia which differ markedly in appearance from one another. On the outer surface of the middle fold is a low epithelium consisting of small cubical cells, 6 to 8 μ in height, whereas the cells lining the inner surface of the outer fold are tall and columnar and form a compact epithelium up to 45 μ thick. The cells of the outer fold contain deeply staining cytoplasm with distally a conspicuous zone of coarse granules (fig. 2, A). The nucleus is situated in the basal half of the cell. These cells appear to be solely responsible for the secretion of the periostracum, as in other bivalves (Kessel, 1944; Brown, 1952; Yonge, 1957). It is noticeable that both the periostracum and the granular zone in the outer fold cells give a positive reaction to the argentaffin test (Lison, 1953), which has been commonly used to detect polyphenols concerned in the tanning of proteins, whereas the epithelial cells of the middle fold are unaffected (fig. 3, A). Although in *Anodonta*, as in certain other lamellibranchs, the periostracum adheres to the epithelium of the middle fold (fig. 3, A), there is no evidence that these cells take any part in periostracum formation (Kessel, 1944).

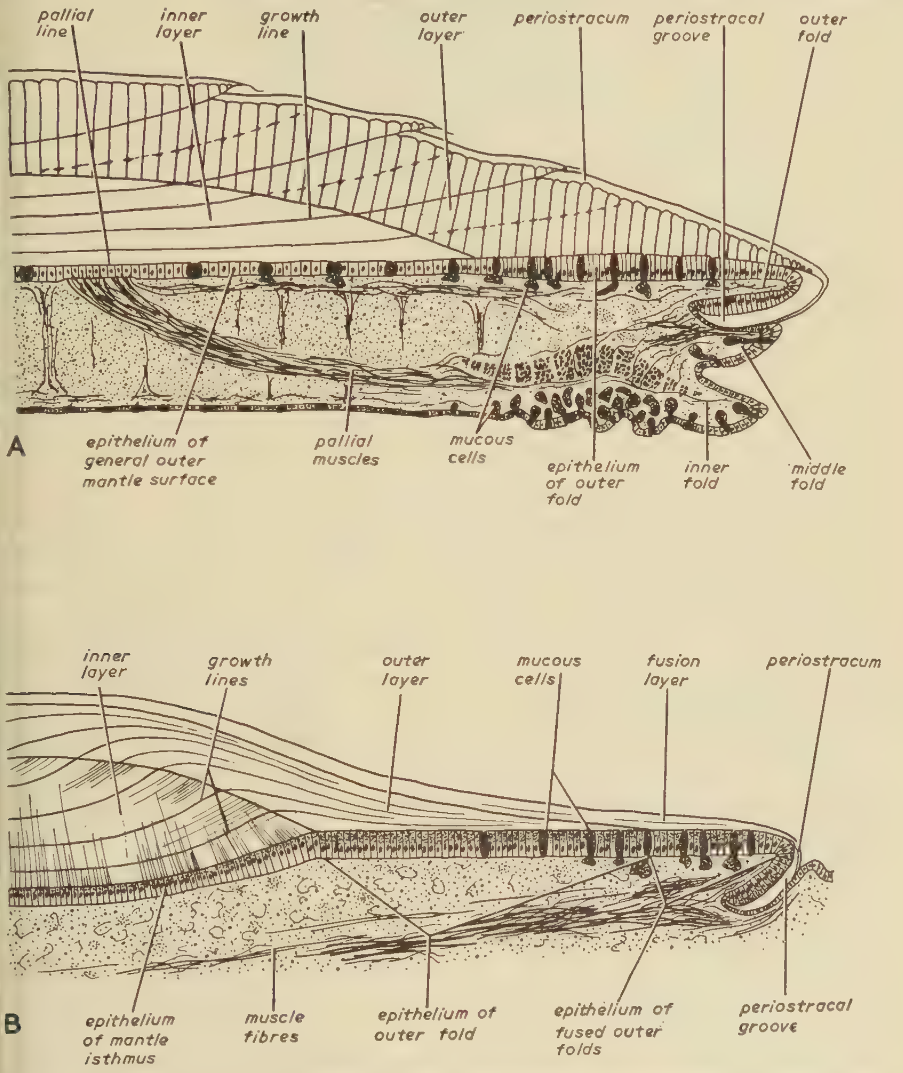


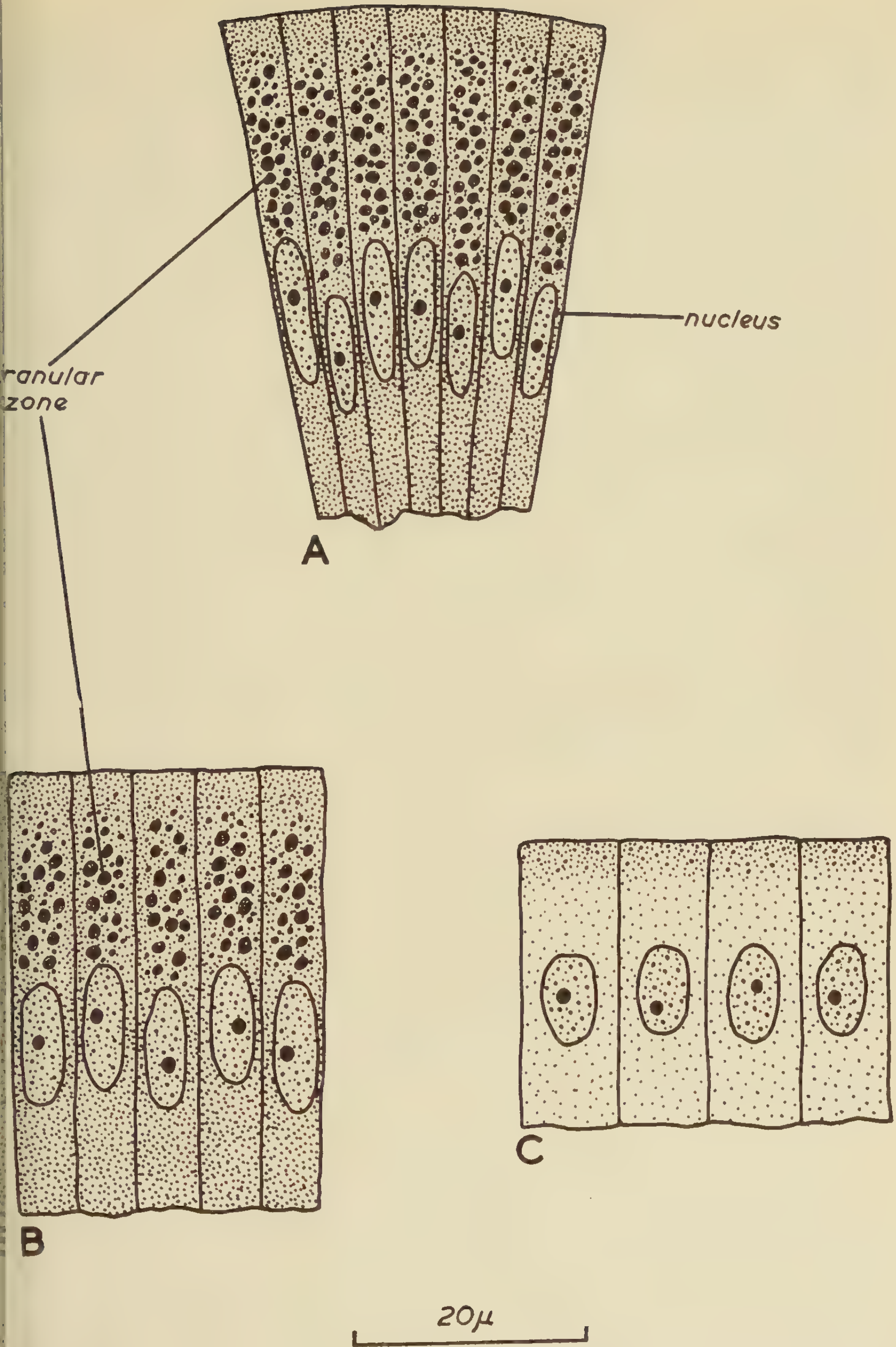
FIG. 1. Diagrams to show the structure of the mantle and its relationship to the shell in *A. cygnea*. A, transverse section of the mantle and valve margin. B, longitudinal section of the posterior end of the ligament and the underlying mantle. Not to scale.

The outer surface of the mantle underlying the valves is divisible into different regions concerned with the secretion of the outer and inner calcareous layers. The outer layer is formed at the mantle edge by the epithelium on the outer surface of the outer fold and the inner layer is secreted by the cells of the general outer surface of the mantle (Owen, Trueman, and Yonge, 1953) (fig. 1, A). According to Yonge (1953), these two regions of the mantle epithelium are separated in most lamellibranchs by the line of attachment of the pallial muscles. In *A. cygnea*, on the contrary (fig. 1, A), as in certain other species, including *A. cellensis* (Siebert, 1913), *Tellina tenuis* (Trueman, 1942) and *M. edulis* (Field, 1922; White, 1937), the pallial muscles are inserted directly on the inner calcareous layer, which is secreted consequently both outside and inside the pallial line. However, the epithelial regions on the outer mantle surface can readily be distinguished from each other histologically. The nucleus and cytoplasm of the epithelial cells underlying the outer layer are essentially similar in structure to those which secrete the periostracum, although the zone of granular inclusions is less prominent (fig. 2, A, B). The epithelium varies in height from 20 to $45\,\mu$ but it may be $55\,\mu$ thick in the region of most rapid shell deposition. In contrast, the cells which secrete the inner layer are broader and usually less tall than those of the outer fold. They contain weakly staining cytoplasm with no definite granular zone and the nucleus is situated centrally (fig. 2, C).

Before comparing the epithelial zones underlying the ligament with those which secrete the valves, it is necessary to consider briefly the general morphology of the ligament and its relationship to the mantle. Although it is well known that the ligament in *Anodonta*, as in most other lamellibranchs, consists of an outer non-calcareous layer and an inner calcified layer (Müller, 1885; de Villepoix, 1892; Rassbach, 1912), there are a number of important features hitherto undescribed.

The ligament is opisthodetic and extends during growth in a posterior direction. Consequently, the relationship between the posterior end of the ligament and the underlying mantle as revealed in longitudinal section (fig. 1, B) is directly comparable with that between the valves and mantle shown in a transverse section through the valve margin (fig. 1, A). As already pointed out, the layers of the ligament have the same origins as the corresponding layers of the valves. The periostracum is secreted by the inner surface of the outer marginal fold, although in *Anodonta* this is not recognizable as a separate layer over most of the outer surface of the ligament (fig. 1, B). The outer layer of the ligament is formed by the outer surface of the outer mantle fold, as is the outer calcareous layer of the valves, while the inner layer of the ligament is secreted by the epithelium of the mantle isthmus in the same manner as the corresponding layer of the valves is produced by the general outer surface epithelium of the mantle (fig. 1, A, B).

These components (periostracum, outer layer and inner layer) constitute the primary ligament which represents the primitive condition of the ligament in the Lamellibranchia (Owen, Trueman, and Yonge, 1953). Usually, however, the primary ligament undergoes some degree of secondary extension. This is the result of fusion of the outer marginal folds at either or both ends of the



IG. 2. Diagrams to show the histological structure, in *A. cygnea*, of the epithelial cells of: A, he inner surface of the outer mantle fold which secrete the periostracum; B, the outer surface f the outer fold underlying the outer calcareous layer of the valve; C, the general outer surface f the mantle underlying the inner layer of the valve. The distal borders of the cells are orientated towards the top of the diagram.

mantle isthmus. As observed by Yonge (1957), the extent of this fusion determines the manner in which the ligament becomes secondarily extended. If fusion involves only the inner surface of the outer mantle folds, then the extension is by way of periostracum. However, in *Anodonta* as in many other lamellibranchs, fusion of the outer surfaces of these folds occurs, resulting in the formation of a fourth component in the ligament, the fusion layer (Owen, Trueman, and Yonge, 1953) (fig. 1, B). This consists of non-calcareous material secreted by the epithelium of the fused outer surfaces of the outer folds (fig. 1, B), i.e. of surfaces which elsewhere along the mantle margin are responsible for the formation of the outer calcareous layer of the valves. In the Lamellibranchia, secondary extension by fusion layer may occur at each end of the primary ligament or exclusively at the posterior end (Yonge, 1957). The former condition occurs in *Anodonta*, although the degree of extension posterior to the primary ligament is comparatively small (fig. 1, B).

The epithelia of the outer fold and fused outer folds are basically similar in appearance (fig. 1, B). This contrasts with the condition described by Yonge (1953) in *Pinna* in which these zones are fairly well defined. Moreover, the constituent cells, which are tall and columnar and form a compact secretory epithelium, are comparable in size and histological structure with those which secrete the periostracum and with the taller of the cells underlying the outer calcareous layer of the valves (figs. 1, B; 2, A, B). The similarity in appearance between these different regions of the outer fold is a characteristic feature of the mantle epithelium in *Anodonta*. The epithelial cells from all these zones have the same properties. In tissues fixed in Bouin's fluid or mercuric-formaldehyde most of the cytoplasm colours pink with Mallory's stain. In material fixed in alcohol, however, the cytoplasm surrounding the nucleus in the basal part of the cell colours deep red, whereas the rest of the cytoplasm including the granular zone colours blue. The granular inclusions react moderately with the mercuric bromphenol blue test for protein (Mazia, Brewer, and Alfert, 1953) and give a positive reaction to the argentaffin test. It is possible that these granules are concerned with the synthesis of the protein component of the shell since they are particularly prominent in the cells of the outer fold which secrete the periostracum and the uncalcified posterior region of the ligament.

As in the epithelium underlying the valves, the epithelial regions which secrete the outer and inner layers of the ligament can be differentiated structurally from one another. The cells of the mantle isthmus differ from those of the outer fold in the appearance of their cytoplasmic contents and the position of the nucleus (fig. 1, B). Only the cytoplasm in the outermost part of the cell stains deeply and the granular zone present in this region is less highly developed. The nucleus is situated in the distal half of the cell. The epithelium is not as tall as that of the outer fold except at the extreme posterior end of the mantle isthmus where the rate of secretion of the inner layer is greatest (fig. 1, B). A characteristic feature of the epithelium of the isthmus is its close connexion to the inner layer of the ligament and to the ridge which separates the ligament from the inner calcareous layer of the adjoining valves. This continuity between the epithelial cells and the matrix of the inner layer, which does not occur between the mantle epithelium and the remainder of the ligament, has previously been observed in species of *Anodonta* by de Villepoix (1892) and also in *T. tenuis* by Trueman (1949).

Mucous tissue is an important constituent of the outer epithelium of the mantle. Mucous cells occur in large numbers in the epithelium outside the pallial line and in that underlying the posterior end of the ligament (fig. 1, A, B). They are also present in the epithelium of the general outer surface of the mantle (fig. 1, A) but do not apparently occur in the mantle isthmus epithelium. The contents of all the mucous cells on the outer mantle surface stain strongly with the periodic acid Schiff reaction for polysaccharides (Hotchkiss, 1948), colour faintly with protein tests, but fail to react with alcian blue (Steedman, 1950) and other tests for acid mucopolysaccharides. According to Pearse (1953), these properties indicate that the mucus is a mucoprotein or a neutral polysaccharide. It may be noted that the mucus secreted by the gland-cells in the inner epithelium of the mantle has a different composition. It colours strongly with alcian blue, shows red (gamma) metachromasia with toluidine blue, and binds with methylene blue in buffer at below pH 4·0, which indicates that it consists of an acid mucopolysaccharide containing sulphate groups. As already observed by Hayashi (1938), calcium salts cannot be detected histochemically in the mucous cells of the mantle in *Anodonta*.

Although the mucus secreted on the outer mantle surface may be involved in the transfer of calcium salts from the epithelial cells to the calcareous layers of the valves, the presence of numerous mucous cells in the epithelia concerned with the deposition of the fusion and outer layers of the ligament, which are completely uncalcified, indicates that this is not its only function. The abundance of mucous tissue at the highly muscular mantle edge (fig. 1 A, B) suggests that the mucus secreted between mantle and shell supplies the necessary lubrication for the withdrawal and re-extension of the mantle margin. Yonge (1953) postulates a similar function for glandular tissue in the outer epithelium of the mantle in *Pinna carnea*. The absence of mucous cells from the epithelium of the mantle isthmus in *Anodonta* may be correlated with the close association between the epithelial cells and the matrix of the inner ligament. This epithelium appears to be unable to move freely on the under surface of the ligament which renders lubrication unnecessary.

M. edulis and *O. edulis*

The epithelia lining the periostracal groove in *M. edulis* are very similar in general appearance to those in *Anodonta*. The periostracum is closely attached to an ill-defined low epithelium on the outer surface of the middle fold, whereas the epithelium on the inner surface of the outer fold consists of a compact layer of tall columnar cells with a basal nucleus and deeply staining cytoplasm in which there is a granular zone in the distal region of the cell. As in *Anodonta*, the histological structure of these epithelia indicates that the periostracum is secreted by the epithelium of the outer fold and not by tha of the middle fold (Kessel, 1944; Brown, 1952).

In *O. edulis*, the distinction between the epithelia lining the periostraca groove is noticeable only in the extreme basal part of the groove (fig. 3, B). In this region, which has been termed the 'periostracal gland' in *O. cucullat* (Awati and Rai, 1931), the epithelium on the inner surface of the outer fol is modified and consists of tall columnar cells with deeply staining, granula cytoplasm (fig. 3, B). These cells secrete a fairly large mass of non-calcareou material which consists of thin laminae lying parallel to the surface of th epithelium. The laminae are continuous with the periostracum which extend towards the mouth of the periostracal groove. It is probable that the periostracum of *O. edulis*, which is extremely thin and hyaline and very elasti (Korringa, 1951), is secreted principally by the epithelium of the outer fol in the depth of the groove and, unlike that in *Anodonta* and *Mytilus*, is no thickened appreciably by the rest of the epithelial cells lining the inner surfac of the outer fold.

The outer epithelium of the mantle of *O. edulis* can be differentiated int zones which secrete the outer and inner calcareous layers of the valve. Th former is 35 μ tall in places and consists of cells which contain more deepl staining and more granular cytoplasm than those of the relatively low epithelium, 8 to 10 μ in height, which extends over the general outer surface o the mantle. In *M. edulis*, however, the difference in appearance between th epithelia underlying the outer and inner layers of the valve is much less noticeable. Apart from a zone of very tall cells at the extreme mantle margin, th epithelium which secretes the outer layer is 10 to 15 μ tall and graduall merges into the very low epithelium, 4 to 6 μ in height, underlying the inner layer. The whole of the outer epithelium of the mantle consists of cells with weakly staining, finely granular cytoplasm, and consequently, unlike the situation in *Anodonta*, the epithelium underlying the outer calcareous layer differ in appearance from that which secretes the periostracum.

Gland-cells occur in the mantle epithelium of both *Ostrea* and *Mytilus*. They are small and infrequent in the epithelium underlying the calcareous

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FIG. 3 (plate). A, transverse section of the mantle edge of *A. cygnea*, in the region of the periostracal groove. The argentaffin test (Lison, 1953) has been applied. The periostracum and the granular zone in the outer fold cells give a strong reaction. Background coloration is due to slightly prolonged treatment. Fixed in Bouin.

B, transverse section of the mantle edge of *O. edulis* to show the structure of the periostracum and the mantle in the region of the periostracal groove. Fixed in mercuric-formaldehyde, stained by Masson's tricolor method.

C, transverse section through the mantle edge of *A. cygnea* to show the localization of alkaline phosphatase in the mantle tissues in the region of the periostracal groove. In this section and in other alkaline phosphatase preparations illustrated in fig. 4, sites of phosphatase activity are indicated by black deposits. Unless otherwise stated, background coloration is due to counterstaining. This preparation shows that there is a high concentration of alkaline phosphatase in the epithelium of the outer mantle fold, whereas little or no enzyme activity occurs in that on the outer surface of the middle fold which lines the periostracal groove. Incubation was for 6 h; counterstained with eosin.

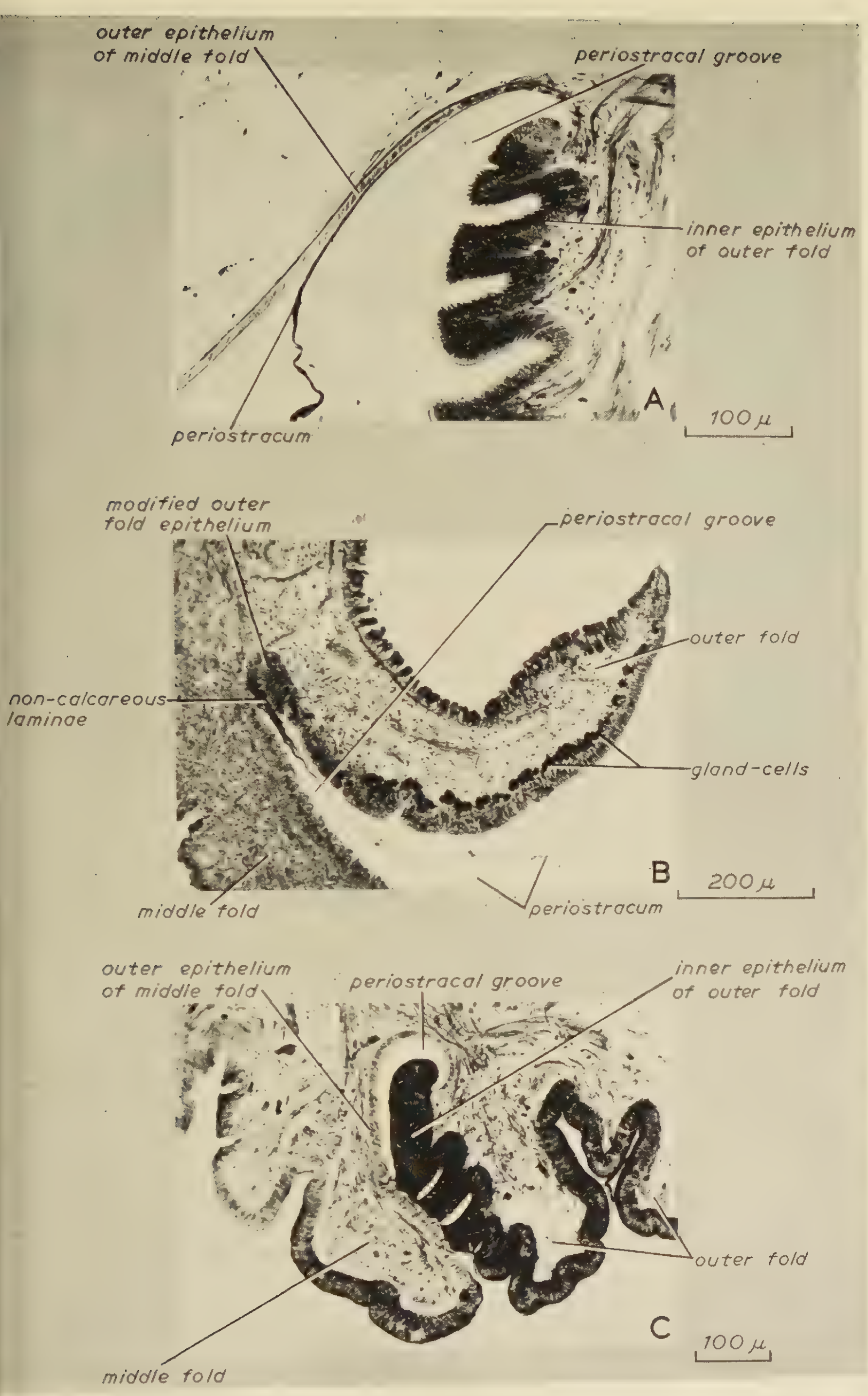

FIG. 3

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ayers of the valve but are large and conspicuous, particularly in *Ostrea* (fig. , B), in the epithelium on the inner surface of the outer fold. These cells have oarse granular contents which do not react with the periodic acid Schiff test, lcian blue, and other stains for mucin. The granules react strongly with ercuric bromphenol blue, which suggests that they consist mainly of protein. hey are coloured by tests for calcium but the intensity of the reaction varies, articularly in the large gland-cells at the mantle margin. Although the gland-ells on the outer surface of the mantle of *Mytilus* and *Ostrea* may play a part n the calcification of the shell, as suggested by Korringa (1952) for *O. edulis*, he abundance and distribution of these cells at the mantle edge indicates hat their products secreted in these regions may be concerned with lubrica-ion, as in *Anodonta*. Even though the glands do not apparently contain ucopolysaccharides, it is not unlikely that their secretions can act physically n the same way as mucus.

In addition to the variations already described, the epithelia on the outer urface of the mantle of *Mytilus* and *Ostrea* differ in one noticeable feature rom those of *Anodonta*, namely that the epithelial regions underlying the outer nd inner layers of the ligament can be distinguished in size and histological ppearance from the zones which secrete the corresponding layers of the alves. The cells which secrete the outer and inner ligament are extremely tall n places and they contain deeply staining, granular cytoplasm. Gland-cells ould not be detected. The epithelium underlying the outer layer of the liga-ent in *Mytilus* is up to 170 μ in height. It passes through a short transitional one into that of the mantle isthmus, 30 to 35 μ tall, which, as in the corre-ponding region of *Anodonta*, consists of cells in which only the distal region ontains deeply staining cytoplasm. In *Ostrea*, the difference in appearance etween the epithelial zones is less marked and both consist of closely packed olumnar cells approximately 100 μ in height.

HISTOCHEMICAL PROPERTIES OF THE MANTLE EPITHELIUM

Certain histochemical tests were carried out on the mantle tissues of a umber of lamellibranchs in order to determine if the regional variations in the tructure of the outer epithelium of the mantle described above are accom-anied by differences in chemical properties between the cells of the various ones. These observations are limited to a description of the localization of hree compounds, alkaline phosphatase, ribonucleic acids, and glycogen, hich have been associated individually or collectively with the physiology f shell deposition (e.g. Bevelander, 1952; Love and Frommhagen, 1953; Kado, 1954), or with bone formation (e.g. Robison, 1923; Glock, 1940; Horowitz, 1942).

Alkaline phosphatase

The localization of alkaline phosphatase in the mantle tissues was investi-ated by Danielli's (1946) modification of the histochemical technique esigned by Gomori (1939). Material was fixed in 80% or absolute alcohol and cleared in benezene, these stages being carried out at 0° C. The tissue were embedded directly in paraffin wax (three changes) in less than 2 h Sections were incubated in the sodium glycerophosphate medium for up t 24 h at 37° C and controls were treated in incubation media lacking th glycerophosphate.

The distribution of alkaline phosphatase in the region of the periostraca groove of *Anodonta* is illustrated in fig. 3, C. There is a high concentration c the enzyme in the epithelium on the inner surface of the outer fold wherea there appears to be no phosphatase activity in that on the outer surface of th middle fold. If the phosphatase present in this region is concerned in th secretion of the periostracum, its localization fully supports the view that it i the inner epithelium of the outer fold which is solely responsible for periostracum formation.

The regional localization of alkaline phosphatase in the epithelium on th outer surface of the mantle of *Anodonta* is particularly interesting. The epithelium on the outer surface of the outer fold underlying the outer calcareou layer of the valve gives a strong phosphatase reaction (fig. 4, A). Enzym activity decreases in the outer epithelium away from the mantle edge and littl or no phosphatase can be detected in the cytoplasm of the cells of the genera outer surface of the mantle which secrete the inner calcareous layer (fig. 4, A). The relative distribution of phosphatase in the epithelium underlying th ligament is almost identical (fig. 4, B). The enzyme occurs in high concentration in the epithelium of the outer fold and fused outer folds which secret the outer and fusion layers of the ligament respectively, whereas phosphatas could not be located in the epithelial cells of the mantle isthmus underlyin the inner layer of the ligament. Consequently there is a sharp line of demarcation, with respect to phosphatase localization, between the epithelial zone underlying the different regions of the ligament (fig. 4, B). These results sho that the histological differences between the epithelium of the outer fold an

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FIG. 4 (plate). A, alkaline phosphatase preparation of a transverse section through the mantl margin of *A. cygnea*. Phosphatase activity is high in the epithelium of the outer mantle fol but decreases markedly along the general outer surface of the mantle. Incubation was for 24 h not counterstained. Background coloration is due to prolonged incubation.

B, longitudinal section of part of the mantle underlying the ligament in *Anodonta* to sho the extreme difference in alkaline phosphatase activity between the epithelium of the oute fold and that of the mantle isthmus. Incubation was for 4 h; not counterstained.

C, localization of alkaline phosphatase in the epithelium of the outer mantle fold in *Anodonta* Enzyme activity is high in the outer cytoplasmic border of the epithelial cells but is only sligh in the mucous cells. Incubation was for 7½ h; counterstained with light green.

D, alkaline phosphatase preparation of a transverse section through the mantle margin o *Cardium edule*. The distribution of the enzyme in the mantle epithelium is very similar to tha in *Anodonta* illustrated in A. Incubation was for 6 h; counterstained with light green.

E, F, sections to show the difference in basiphilia between the cytoplasm of the epithelia cells of the outer mantle fold in *Anodonta* before and after treatment with ribonuclease. E control section; F, section digested with ribonuclease. Both sections fixed in neutral formaldehyde solution, stained aqueous toludine blue.

G, localization of glycogen deposits (indicated by arrows) in the epithelium of the genera outer surface of the mantle of *Anodonta*. Stained with Best's carmine, counterstained wit haemalum.

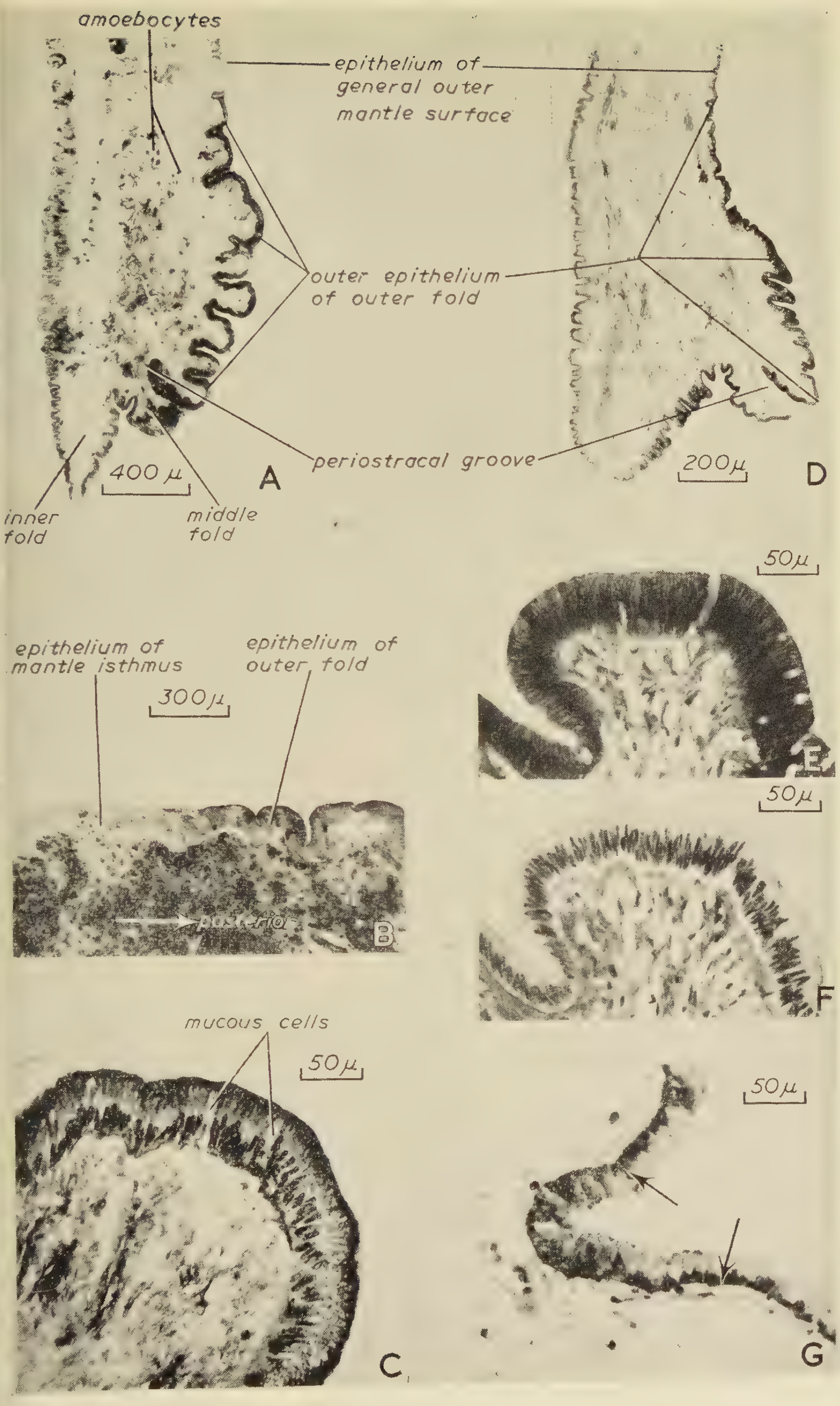

FIG. 4

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at of the rest of the outer surface of the mantle of *Anodonta* are associated ith a marked difference in alkaline phosphatase activity.

The detailed localization of alkaline phosphatase in the epithelium of the uter mantle fold is shown in fig. 4, c. The enzyme is located mainly in the pithelial cells and there appears to be little or no activity in the mucous cells. he highest concentration of phosphatase occurs in the outermost part of the pithelial cell (fig. 4, c), although the reaction spreads farther into the main ody of the cell after long periods of incubation. The nucleus also gives a ositive response to the phosphatase test, the reaction occurring mainly in e nucleolus although there may be some enzyme activity in the region of the uclear membrane. It is possible, however, that the nuclear reaction may be n artifact caused by the high concentration of phosphatase in the distal egion of the cell. Jacoby and Martin (1949) have shown that almost inactive uclei may give a positive test for phosphatase owing to diffusion of the nzyme from adjacent sites of high phosphatase activity. In addition to its resence in the outer epithelium of the mantle, alkaline phosphatase also ccurs in certain amoebocytes in the connective tissue and in epithelial cells f the middle fold outside the periostracal groove, the inner fold, and part of e inner surface of the mantle at the mantle margin (figs. 3, c; 4, a).

Although specimens of *M. edulis* were examined at different times of the ear, alkaline phosphatase could not be detected with certainty in any part of e mantle epithelium. Previous work on *Mytilus* has produced conflicting esults. Manigault (1939) and Bourne (1943) claim to have demonstrated lkaline phosphatase in the mantle tissues whereas Roche and Berne (1951) btained negative results. In other lamellibranchs investigated, however, lkaline phosphatase can be readily detected histochemically in certain regions f the mantle epithelium. Phosphatase occurs in the epithelia lining the periostracal groove of a number of bivalves and in *Cardium edule* its localization is dentical with that in *Anodonta*, the enzyme being confined to the epithelium n the inner surface of the outer fold (fig. 4, d). In *O. edulis* and *Pecten maximus*, on the other hand, phosphatase can be located in the epithelia on both ides of the groove, although in *Ostrea* it appears to be present in relatively igher concentration in the tall epithelium of the outer fold which secretes the ulk of the periostracal material at the base of the groove.

In a number of species, the localization of alkaline phosphatase in the pithelium which secretes the valve shows similarity to that occurring in *Anodonta*. This resemblance is particularly well illustrated in *C. edule* in which phosphatase activity is considerably higher in the tall epithelial cells at he mantle edge than in the epithelium of the general outer surface of the mantle (fig. 4, d). The enzyme can also be detected in relatively greater concentration in the epithelium at the mantle margin of *T. tenuis*, *Nucula sulcata*, *P. maximus*, and *O. edulis*, although in *Ostrea* the distinction between the pithelial zones is usually less well marked and phosphatase occurs in appreciable quantities in the cells of the general outer mantle surface. Alkaline phosphatase can be demonstrated in the epithelium which secretes the ligament in certain of the bivalves investigated but its localization differs from th described for the corresponding region in *Anodonta*. The enzyme is foun in all regions of the epithelium underlying the ligament in *Cardium* and *Ostre* and in the latter, activity tends to be higher in the epithelium of the mant isthmus than in that of the outer mantle fold. In *Tellina*, however, alkalin phosphatase could not be detected in either of these regions.

Ribonucleic acids

The ribonucleic acid content of the epithelial cells of the mantle was studie by comparing the degree of basiphilia of the cytoplasm before and after trea ment with a solution of purified crystalline ribonuclease. The ribonucleas test has been subjected to criticism on certain grounds (Danielli, 1947), bu as pointed out by Davidson (1953), it can provide useful information if th results are interpreted with caution. Sections of mantle tissue fixed in alcoho or formaldehyde solution were incubated for 1 h at 37° C in a 0·1% solution o ribonuclease in distilled water or phosphate buffer at pH 7·0, which had bee heated previously at 80° C for 15 to 20 min to remove any traces of proteolytic enzymes (Bradfield, 1951; Pearse, 1953). Incubated sections and control slides treated with distilled water or buffer only were stained in a 0·2% aqueous solution of toluidine blue and mounted in Canada balsam after rapi dehydration in acetone and clearing in xylene.

Before treatment with ribonuclease the cytoplasm of the epithelial cells o both outer and inner surfaces of the outer mantle fold in *Anodonta* stain strongly with toluidine blue (fig. 4, E). However, the degree of basiphilia varie in different regions of the cell. It is strongest in the cytoplasm surroundin the nucleus in the basal half of the cell and is least in that zone at the oute border which exhibits the most intense alkaline phosphatase activity. Thi distinction is particularly well marked in alcohol-fixed tissues in which th region showing strong basiphilia corresponds with that which colours dee red with Mallory's stain. After incubation with ribonuclease this basiphilia removed almost completely from the cell contents and only parts of the nucleu continue to show affinity for toluidine blue (fig. 4, F). This suggests that th cytoplasm in the basal region of the cells of the outer fold is rich in ribonucle acids. In contrast, the ribonucleic acid content of the epithelial cells of the re of the outer surface of the mantle appears to be appreciably lower. The cytoplasmic contents of the cells which secrete the inner calcareous layer of th valves exhibit very slight basiphil properties and the epithelium of the mantl isthmus only contains noticeable quantities of ribonucleic acids in the cytoplasm in the distal region of the cells.

The localization of ribonucleic acids in the outer mantle epithelium suggests that they may be concerned in the secretion of the protein component o the non-calcareous material (conchiolin) present in the shell. They appear t occur in high concentration in the epithelial cells which secrete the periostracum and the posterior uncalcified region of the ligament. It is well know that a high content of ribonucleic acids exists in cells that synthesize protein many vertebrate tissues (Caspersson, 1947; Davidson, 1949), and in the silk ands of invertebrates (Bradfield, 1951). The ribonuclease test was applied the mantle tissues of other lamellibranchs with comparable results. The bonucleic acid content of the outer epithelium varies in different regions of e mantle and appears greatest in the epithelial cells which produce relatively rge amounts of non-calcareous material, as for example in those which secrete e periostracum in *Mytilus* and the ligament of *Mytilus* and *Ostrea*.

It is noticeable that epithelial cells on the outer mantle surface which have rich ribonucleic acid content may also show high alkaline phosphatase tivity. The demonstration of alkaline phosphatase in epithelia which pro- uce completely non-calcareous material, including those which secrete the riostracum and the outer ligament in *Anodonta*, *Ostrea*, and *Cardium*, dicates as already pointed out by Kado (1954) that the enzyme may take rt in conjunction with ribonucleic acids in the synthesis of the protein mponent of the shell. Attempts have been made to correlate phosphatase ith the secretion of calcium salts and the deposition of the mineral component the valves (e.g. Manigault, 1939; Bevelander and Benzer, 1948; Love and rommhagen, 1953), but these observations do not account for the occurrence the enzyme in epithelial cells which secrete uncalcified regions of the shell. lthough a variety of possible functions has been ascribed to alkaline phos- hatase, there is a considerable amount of evidence to suggest an association phosphatase with nucleic acid metabolism and/or the synthesis of fibrous roteins (Fell and Danielli, 1943; Bradfield, 1950, 1951; Danielli, 1953, 1954). he properties of the epithelial cells of the outer mantle fold in *Anodonta*, which ribonucleic acids appear to be more highly concentrated in the asal part of the cell whereas phosphatase activity is highest at its outer border, rrespond closely with those exhibited by the cells that secrete protein in e silk glands of certain arthropods, as described by Bradfield (1951). Brad- eld considered that 'the phosphatase may be part of a system of enzymes volved in liberating the finished protein from a complex with nucleic acids'. owever, as observed by Roche and Berne (1951), the existence of a relation- hip between alkaline phosphatase and the synthesis of fibrous proteins should ot be regarded as universal since alkaline phosphatase can be detected in gions of the mantle other than the epithelia concerned solely with the secre- on of shell protein.

lycogen

The distribution of glycogen in the mantle was investigated on material xed in Rossman's fluid (a mixture of 90 ml of a saturated solution of picric cid in absolute alcohol with 10 ml of neutral formalin) for 24 h at 0° C Kado, 1953). After embedding and sectioning in the normal manner, glyco- en was identified by staining with Best's carmine (Gurr, 1953), or by the eriodic acid Schiff reaction. Controls were similarly stained after prior eatment with a solution of diastase at room temperature.

Apart from appreciable amounts in the connective tissue, the most striking feature of the distribution of glycogen in the mantle of *Anodonta* is t presence of large deposits in the epithelial cells of the general outer surfa of the mantle which secrete the inner layer of the valve (fig. 4, G). Masses glycogen occur in the basal part of these cells and in some they extend throug the body of the cell to the external surface. Near the mantle margin t deposits diminish in size, and in most of the epithelial cells of the outer fo glycogen is absent, or it occurs only in the form of small granules in t cytoplasm. Similarly, the epithelia of the outer fold which secrete the peri stracum and the posterior part of the ligament contain very small quantiti of glycogen. The epithelium of the mantle isthmus may contain a fairly lar number of glycogen granules in the proximal region of the cells but t deposits are much less extensive than in the cells of the general outer surfa of the mantle.

Similar results were obtained with other bivalves. The most consta feature of comparison with *Anodonta* is the presence of large deposits of glyc gen in the epithelium of the general outer surface of the mantle. This conditio occurs, for example, in *Ostrea*, *Mytilus*, *Tellina*, and *Nucula*. Moreover, as *Anodonta*, the cells principally concerned with the secretion of conchioli including those which secrete the periostracum of *Mytilus* and the ligament *Mytilus* and *Ostrea*, contain little or no glycogen. It may be noted that the does not appear to be a simultaneous occurrence of glycogen and high alkalin phosphatase activity, as has been demonstrated in certain vertebrate tissu in which calcification is taking place (Glock, 1940; Horowitz, 1942). Glycoge deposits are most conspicuous in the epithelial cells underlying the inner lay of the valves in which little phosphatase can be detected histochemically. Th agrees with observations made by Kado (1954), who showed that regions the mantle of *O. gigas* and *Venerupis semidecussata* which contain glycoge give only a weak reaction for alkaline phosphatase.

DISCUSSION

The epithelial regions of the mantle concerned with the secretion of t different layers in the lamellibranch shell are often well defined, although t extent of this differentiation may vary considerably. Variations in size histological structure between epithelial cells underlying different layers the shell occur in *A. cygnea*, *O. edulis*, and *M. edulis* and have been recorde previously in other bivalves, including species of *Pinna* (Yonge, 1953) and *tenuis* (Trueman, 1949). The results of histochemical tests on the mant suggest that these variations in cell structure are accompanied by differenc in chemical properties. Alkaline phosphatase activity, for example, vari appreciably in different regions of the mantle and in many bivalves it is muc higher in the epithelial cells underlying the outer layer of the valves than in t epithelium of the general outer mantle surface. In contrast, the cells constitu ing the latter are distinguished from those at the mantle edge by a particular high glycogen content.

The relative thickness of the epithelia in different regions of the mant

ay be correlated with the rate with which they secrete shell material. Thus,
e considerable difference in height of the epithelia underlying the outer
d inner layers of the ligament in *Mytilus* probably reflects the much greater
eed with which the outer ligament is secreted. Another feature for dis-
guishing between epithelial cells from different areas of the mantle is the
tensity of staining of their cytoplasmic contents. Although other factors
ay be involved, this feature may well be determined by the relative amount
non-calcareous material which the cells secrete. The periostracum in
ytilus, which constitutes three-quarters of the total valve conchiolin (Lop-
ns, 1920), is secreted by epithelial cells with deeply staining, granular
toplasm, whereas the cells underlying the calcareous layers have sparse cyto-
asmic contents which colour only faintly with stains. A somewhat similar
stinction has been made by de Villepoix (1892), who observed two types
epithelium in the mantle of *Anodonta* and *Mytilus*, namely that which
cretes calcium ('l'épithélium calcigène') and that which produces non-
lcareous material ('l'épithélium chitinogène'). It is interesting to note that
ithelial cells which have deeply staining cytoplasm appear to contain
preciable quantities of ribonucleic acids. The cytoplasm of cells concerned
incipally with the secretion of the protein component of the shell stains
ongly with toluidine blue and this basiphilia is greatly reduced after extrac-
n with ribonuclease.

The degree of differentiation of the outer epithelium of the mantle may be
termined by the extent to which the different layers of the shell vary in
mposition. In certain lamellibranchs, including *T. tenuis* (Trueman, 1942)
d *M. edulis*, there is little apparent modification in structure of the epithe-
m underlying the calcareous layers of the valves apart from an increase in
ight at the mantle edge where the rate of shell secretion is greatest. This
ay be related to a lack of sharp distinction in these species between the non-
lcareous constituents of the different valve layers. In the valves of *Tellina*,
ich unlike many freshwater molluscs (e.g. *Anodonta*) contain a very low
oportion of non-calcareous material, the conchiolin components of the
lcareous layers have similar properties and appear to differ in nature from
e periostracum (Trueman, 1949). It will be shown in a later paper that in
ytilus, the conchiolin matrix of the outer calcareous layer exhibits almost the
me chemical properties as that of the inner layer. In other bivalves, as for
ample in *A. cygnea* and *O. edulis*, in which the outer and inner layers of the
lves differ appreciably in composition, the zonation of the outer epithelium
the mantle is more pronounced. Korringa (1951) observed that there is
proximately 4·0% of conchiolin in the outer calcareous layer of the valves
Ostrea, whereas the inner layer contains only 0·5–0·6% of non-calcareous
aterial. Similarly in *Anodonta*, the inner calcareous layer contains a relatively
all amount of conchiolin and it is also known that this material has a
fferent composition from the conchiolin of the outer layer of the valves and
e periostracum (Friza, 1932; Beedham, 1954).

In most lamellibranchs the outer and inner layers of the primary ligament

are formed by epithelia which are distinct histologically from those unde
lying the corresponding layers of the valves (Yonge, 1953). This distinctio
which as shown in the present investigation is well illustrated in *Mytilus* ar
Ostrea, is probably due to a large extent to the higher rate of secretion of non
calcareous material involved in the deposition of the ligament. In *A. cygne*
however, the epithelial regions which secrete corresponding layers of the valv
and ligament are more closely comparable. This is especially noticeable whe
comparing the epithelial zones of the outer mantle fold underlying the out
layers of the valves and ligament. These epithelia are of approximately th
same thickness, which suggests that they secrete shell material at a simil
rate, and are also fundamentally alike both in histological structure and in the
reactions to histochemical tests. The properties of these corresponding epith
lial regions of the mantle may well reflect the particularly close similari
which exists in *Anodonta* between the composition of the non-calcareo
components of the 'outer' layers of the valves and ligament (Beedham, 1954
In addition, although the epithelium forming the inner layer of the ligame
differs histologically from that which secretes the inner calcareous layer of th
valves, both these regions can be distinguished in structure and properti
from the epithelia at the mantle edge. The appearance of the mantle epith
lium in *Anodonta* can readily be correlated with the view expressed by Owe
Trueman, and Yonge (1953) that the outer and inner layers of the ligamen
in the Lamellibranchia are to be regarded as derivatives of the same region
of the mantle as the corresponding layers of the valves.

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REFERENCES

Awati, P. R., and Rai, H. S., 1931. Indian zool. Mem., **3**.
Beedham, G. E., 1954. Nature, **174**, 750.
Bevelander, G., 1952. Biol. Bull., **102**, 9.
—— and Benzer, P., 1948. Ibid., **94**, 176.
Bourne, G., 1943. Quart. J. exp. Physiol., **32**, 1.
Bradfield, J. R. G., 1950. Biol. Rev., **25**, 113.
—— 1951. Quart. J. micr. Sci., **92**, 87.
Brown, C. H., 1952. Ibid., **93**, 487.
Caspersson, T., 1947. Symp. Soc. exp. Biol., **1**, 127.
Danielli, J. F., 1946. J. exp. Biol., **22**, 110.
—— 1947. Symp. Soc. exp. Biol., **1**, 101.
—— 1953. *Cytochemistry: a critical approach.* New York (Wiley).
—— 1954. Proc. Roy. Soc. B, **142**, 146.

AVIDSON, J. N., 1949. Ann. Rev. Biochem., **18,** 155.
— 1953. *The biochemistry of the nucleic acids.* London (Methuen).
ELL, H. B., and DANIELLI, J. F., 1943. Brit. J. exp. Path., **24,** 196.
IELD, I. A., 1922. Bull. U.S. Bur. Fish., **38,** 127.
RIZA, F., 1932. Biochem. Z., **246,** 29.
LOCK, G. E., 1940. J. Physiol., **98,** 1.
OMORI, G., 1939. Proc. Soc. exp. Biol. N.Y., **42,** 23.
URR, E., 1953. *A practical manual of medical and biological staining techniques.* London (Hill).
AYASHI, K., 1938. Annot. zool. Jap., **17,** 95.
OROWITZ, N. H., 1942. J. dent. Res., **21,** 519.
OTCHKISS, R. D., 1948. Arch. Biochem., **16,** 131.
COBY, F., and MARTIN, B. F., 1949. Nature, **163,** 875.
ADO, Y., 1953. J. Sci. Hiroshima Univ. Ser. B, Div. 1, **14,** 259.
— 1954. Ibid., **15,** 183.
ESSEL, E., 1944. Z. Morphol. Ökol. Tiere, **40,** 348.
ORRINGA, P., 1951. Proc. Calif. Acad. Sci., **27,** 133.
— 1952. Quart. Rev. Biol., **27,** 266.
ISON, L., 1953. *Histochimie et cytochimie animales.* Paris (Gauthier-Villars).
OPPENS, K., 1920. Ann. Soc. zool. malac. Belg., **51,** 75.
OVE, R., and FROMMHAGEN, L. H., 1953. Proc. Soc. exp. Biol. N.Y., **83,** 838.
ANIGAULT, P., 1939. Ann. Inst. océanogr. Monaco, **18,** 331.
AZIA, D., BREWER, P. A., and ALFERT, M., 1953. Biol. Bull., **104,** 57.
ÜLLER, F., 1885. Zool. Beitr. Berl., **1,** 206.
WEN, G., TRUEMAN, E. R., and YONGE, C. M., 1953. Nature, **171,** 73.
EARSE, A. G. E., 1953. *Histochemistry, theoretical and applied.* London (Churchill).
ASSBACH, R., 1912. Z. wiss. Zool., **103,** 363.
OBISON, R., 1923. Biochem. J., **17,** 286.
OCHE, J., and BERNE, H. A., 1951. C.r. Soc. Biol. Paris, **145,** 1474.
EBERT, W., 1913. Z. wiss. Zool., **106,** 449.
TEEDMAN, H. F., 1947. Quart. J. micr. Sci., **88,** 123.
— 1950. Quart. J. micr. Sci., **91,** 477.
RUEMAN, E. R., 1942. J. roy. micr. Soc., **62,** 69.
— 1949. Proc. zool. Soc. Lond., **119,** 717.
ILLEPOIX, M. DE, 1892. J. Anat. Paris, **28,** 461.
HITF, K. M., 1937. L.M.B.C. Memoirs, **31,** *Mytilus.*
ONGE, C. M., 1948. Nature, **161,** 198.
— 1953. Phil. Trans. B, **237,** 335.
— 1957. Pubbl. Staz. zool. Napoli, **29,** 151.

The Utilization of I^{131} by Certain Lamellibranchs, with Particular Reference to Shell Secretion

By G. E. BEEDHAM *and* E. R. TRUEMAN

(*From the Departments of Zoology of Glasgow and Hull Universities*)

With one plate (fig 1).

SUMMARY

A study of the utilization of I^{131} by the lamellibranchs *Anodonta cygnea*, *Sphaerium rneum*, and *Mytilus edulis* has been made with particular reference to the secretion the valves and ligament.

While autoradiographs show that I^{131} is localized in the alimentary canal and diges- ve gland, the main distribution of the isotope is on the surfaces of the shell and body posed to the radioactive medium. The iodine associated with the shell occurs princi- lly on the periostracum. There is, however, no positive evidence to show that I^{131} actively incorporated into the shell by the secretory activity of the mantle. The sults are discussed in relation to the ability of other invertebrates to accumulate otein-bound iodine.

INTRODUCTION

N contrast to the skeletons of certain sponges and corals which contain iodinated tyrosine and also traces of thyroxine (Roche, 1952), the structural oteins of molluscan shells appear to contain little or no natural iodine. ameron (1914, 1915), for example, could not detect iodine in the shell of *ya arenaria* although he found 0·042% in the byssus of *Mytilus*. However, the course of recent work on the utilization of radioactive iodine by inverte- rates, Gorbman, Clements, and O'Brien (1954) showed that molluscs are in ct able to accumulate the isotope into the body and that protein-bound dioiodine can subsequently be located both in the shell and in the under- ing mantle. They found, moreover, that in the bivalve *Musculium partumeium*, e isotope can be identified in hydrolysates of the body tissues in the form monoiodotyrosine, diiodotyrosine, and thyroxine.

These observations are of particular interest in view of the fact that tyro- ne is an important constituent of the scleroproteins in the periostracum and e outer shell layers in *Anodonta cygnea* and other lamellibranchs (Roche, anson, and Eysseric-Lafon, 1951; Beedham 1958*b*). If tyrosine compounds belled with radioiodine are utilized in shell formation, it is reasonable to ppose that these particular regions of the valves and ligament will specifically cumulate the isotope. Consequently, it seemed worth while to investigate in eater detail the metabolism of radioactive iodine in the Lamellibranchia ith a view to tracing the secretion of artificially iodinated tyrosine from the antle, in particular from the outer mantle fold, which forms the periostracum d the outer layers of valves and ligament.

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MATERIALS AND METHODS

The general procedures followed in the present investigation are the sam as those adopted by Gorbman, Clements, and O'Brien (1954). Animals wer exposed to the radioactive iodine by immersion in water containing the iso tope I^{131} (carrier-free, as iodide), and the precise localization of iodine in th tissues was subsequently determined by autoradiography.

The lamellibranchs used were *A. cygnea* (small specimens, 4 to 5 cm long) *Sphaerium corneum*, and *Mytilus edulis*. *Anodonta* was employed mainly fo studying the general distribution of iodine in the body after relatively shor periods of exposure. Specimens were immersed in water containing an initia concentration of 1 mC of I^{131} per litre for periods ranging from 24 h to 14 days Small specimens of *Mytilus*, which were primarily used for investigating th metabolism of iodine in relation to the secretion of byssus threads, wer exposed to a similar concentration of the isotope for 1 week.

In addition, long-term experiments were conducted with *Sphaerium* t observe the localization of iodine after appreciable shell formation has bee allowed to take place. *Sphaerium* is particularly useful for experiments of thi kind since it readily secretes new shell material even under laboratory conditions. The animals were given exposure periods of up to 10 weeks in wate containing 200 μC of I^{131} per litre, this concentration being maintained a nearly as possible at a constant level.

After exposure to iodine, the animals were fixed in absolute alcohol o Bouin's fluid and embedded in paraffin wax or ester wax (Steedman, 1947) Decalcification was carried out in 30% formic acid, which is less liable t leach out the radioactive material than the inorganic acids, nitric and hydrochloric (Siffert, 1948). Autoradiographs were prepared by the stripping fil technique of Pelc as described by Pearse (1953). They were developed i Ilford D 19B or amidol and the sections counterstained with haemalum According to Gorbman, Clements, and O'Brien (1954), only the protein-bound form of radioiodine would be expected to remain in the sections afte the procedures involved in the preparation of material for autoradiography.

OBSERVATIONS

Anodonta cygnea

The most noticeable feature shown on autoradiographs of the body o *Anodonta* is the accumulation of iodine along the surfaces of the organs withi the mantle cavity. The isotope is localized on the inner epithelium of th mantle, and on the epithelia of the gills, labial palps, and much of the foot The concentration of iodine on the inner surfaces of the palps is particularl intense (figs. 1, A, B). The radioiodine associated with the gills occurs mainl on the outer surfaces of the filaments, especially in the region of the latera and latero-frontal cilia, although moderately strong autoradiographs are als given by the remainder of the filament surfaces and by the epithelia linin the interlamellar spaces and the exhalant chamber. It is interesting to observ

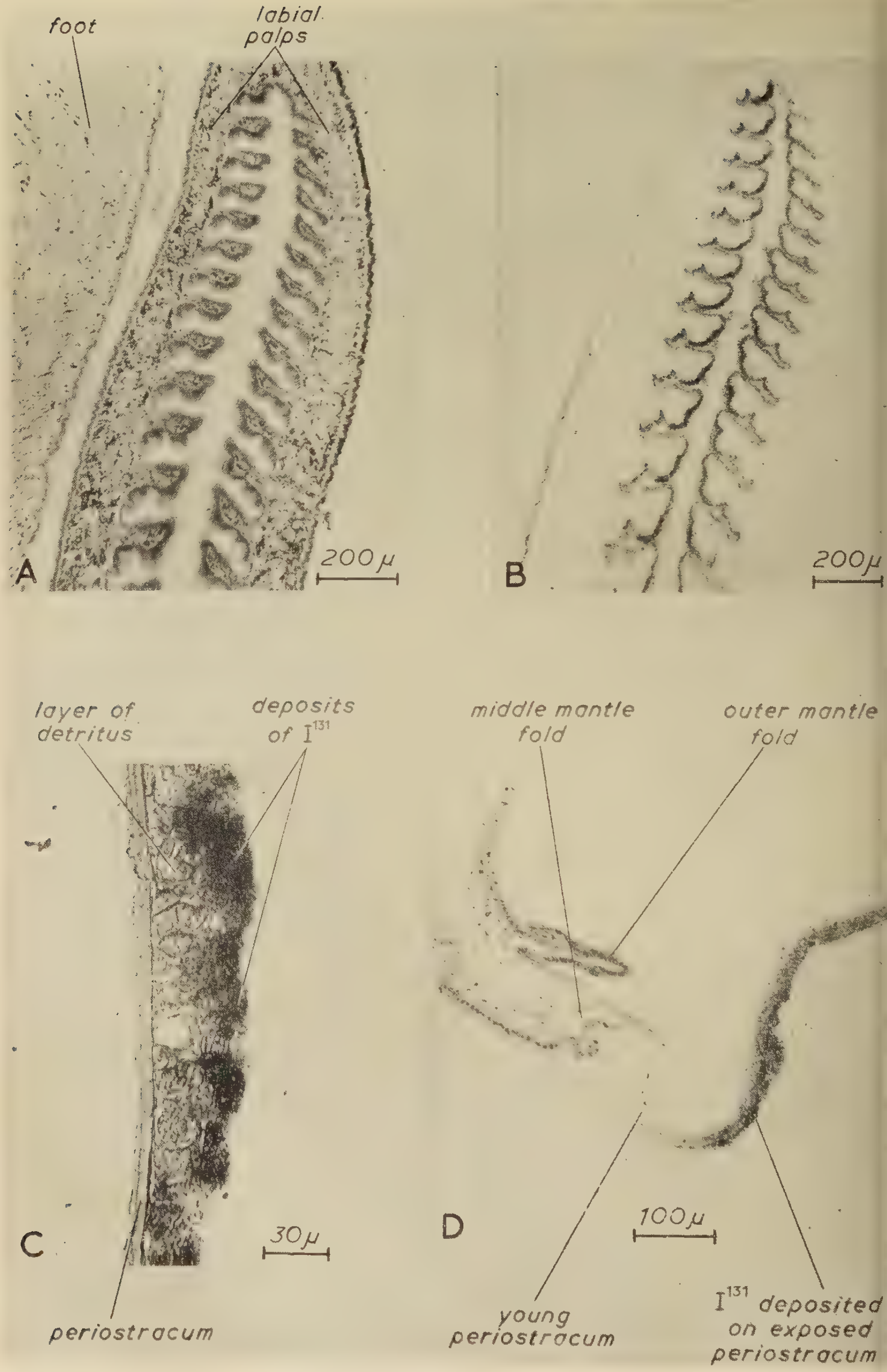

FIG. 1

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hat the areas of the mantle and foot which are usually in direct contact with he external medium show much less tendency to concentrate iodine than the egions facing the mantle cavity. Negligible amounts of I^{131} occur on the iddle and inner marginal folds of the mantle (i.e. the pallial curtain) and n the tip of the foot which normally protrudes between the ventral margins f the valves.

This distribution of the isotope suggests that a positive mechanism exists or the direct uptake of iodine by the epithelia lining the mantle cavity. Auto-adiographs show that the iodine is mainly localized on the external borders f these epithelia, although it probably also occurs to a lesser extent in the ytoplasm in the outermost part of the epithelial cells. It has already been hown that radioactive calcium can be absorbed directly from the surrounding edium by the mantle, foot, and gills of *A. cygnea* (Schoffeniels, 1951), and y the mantle in mantle shell preparations of *Crassostrea virginica* (Jodfrey, 953). Pomeroy and Haskin (1954) found that labelled phosphate ions enter he oyster principally by way of the gills, and they observed that this may also e the main source of calcium for shell desposition. However, there is little oubt that radioiodine also enters the body from the digestive system. ppreciable concentrations of the isotope can be detected on autoradiographs f the digestive gland. These deposits are often diffuse but in some specimens he iodine is found to be concentrated both in the lumen and in the large acuolated cells of the tubules of the digestive diverticula. The isotope can lso be located in the lumen of the stomach and intestine but it is not known hether iodine passes through the epithelia lining these parts of the gut.

Except for the accumulation in the digestive gland, the amount of iodine emonstrable by autoradiography within the body is small. After absorption, he iodine is probably dispersed throughout the tissues by the haemocoel, but t does not appear to be stored in any part of the body. Little or no iodine ould be detected in the mantle, apart from the deposits occurring on its nner surface. The epithelia concerned with the deposition of the periostracum and the different layers of the valves and ligament consistently give egative autoradiographs.

FIG. 1 (plate). A and B, distribution of I^{131} in the region of the labial palps in *Anodonta cygnea*. , stained section of foot and labial palps of *A. cygnea*. B, an autoradiograph which has been tripped from the section illustrated in A. The autoradiograph shows the strong localization f radioiodine on the inner surfaces of the palps, with weaker deposits on the outer epithelia f the palps and on the surface of the foot.

C, section of a small portion of the decalcified shell of *A. cygnea* with attached autoradio-raph to show the large deposits of I^{131} adsorbed on the layer of detritus occurring on the uter surface of the periostracum. The underlying conchiolin matrix of the shell is not shown.

D, transverse section through the mantle and valve margin of *Sphaerium corneum*, showing n the overlying autoradiograph the variation in distribution of I^{131} between different regions f the periostracum. Whilst there is a strong localization of iodine on the periostracum which overs the outer shell surface and is directly exposed to the surrounding radioactive medium, he young, newly secreted periostracum has not accumulated the isotope. It may be assumed hat the latter portion would normally be covered by the outer and middle marginal folds of he mantle, which in the section are shown retracted as a result of fixation, thus preventing he young periostracum from having immediate access to the isotope.

The relationship of radioiodine to the non-calcareous material (conchiolin) in the shell is particularly interesting. Autoradiographs show that iodine becomes deposited over the whole outer surface of the shell. Large concentrations of the isotope occur both on the periostracum which superficially covers the valves and on the fusion layer which, because the periostracum becomes worn away in the hinge region, extends over much of the external surface of the ligament (Beedham, 1958*a*). It should be noted, however, that the iodine is only associated directly with these layers where they are in actual contact with the surrounding medium. As illustrated in fig. 1, C, the isotope accumulates equally well on the detritus which tends to form an irregular layer over large areas of the shell. If the shell is coated with celloidin before exposure to the isotope, the iodine is similarly deposited on this artificial covering and does not affect the periostracum. Although it is possible that the I^{131} occurring on the periostracum and fusion layer may be chemically combined with the protein at the surfaces of these layers, it seems unlikely that the radioiodine associated with the extraneous matter can be in a protein-bound form. Since carrier-free isotopes tend to be strongly adsorbed on any solids with which they come into contact (Francis, Mulligan, and Wormall, 1954), it may be suggested that a similar factor is at least partly responsible for the accumulation of I^{131} on the shell. This adsorption may well be sufficient to withstand the techniques employed in the preparation of autoradiographs.

In general the iodine associated with the shell is localized only on the surfaces which have direct access to the isotope. With the exception of one specimen, in which small deposits of iodine could be detected on the under surface of the outer calcareous layer near the margin of the valves, the conchiolin of the outer and inner layers of both valves and ligament was found to give negative autoradiographs. Owing to interference from the radioiodine deposited on the outer surface of the shell, it is difficult to assess the precise localization of the isotope on autoradiographs of the margins of the valves and ligament. However, there is no positive evidence from these sections that I^{131} becomes actively incorporated either into the periostracum or into the fusion layer. The amount of iodine occurring on the periostracum certainly diminishes in extent towards the extreme mantle edge, especially in the region of the periostracal groove where this layer is secreted.

Sphaerium corneum

The results of the experiments on *Sphaerium* are similar in many respects to those obtained for *Anodonta*. Despite the fact that shell formation occurred in the presence of radioiodine, the isotope could not be detected either in the non-calcareous matrix of the shell or in the underlying mantle tissues. Apart from small local concentrations of iodine in the lumen of the alimentary canal, the isotope is scantily distributed throughout the body. The strongest autoradiographs of specimens examined after either 2 or 10 weeks are invariably given by the external surfaces of the valves and ligament. However, although the iodine becomes deposited over much of the periostracum it does not pear to accumulate in the portion most recently secreted (fig. 1, D). There is sharp demarcation line on autoradiographs between the youngest part of e periostracum which lies protected within the folds of the mantle edge d the remainder of the periostracum which is exposed to the surrounding edium (fig. 1, D).

ytilus edulis

As in *Sphaerium*, the radioiodine distributed on the shell occurs only on e external surface of the periostracum which is in direct contact with the ter containing the isotope. At its origin, the periostracum gives negative toradiographs, as does the remainder of the shell conchiolin and also the antle. It is noticeable that the localization of iodine in relation to the byssus reads is essentially the same as that associated with the shell. As found by orbman, Clements, and O'Brien (1954), newly formed byssus threads autodiograph strongly. Nevertheless, the threads only begin to accumulate dine as they are secreted into the posterior groove of the foot and become lly exposed to the radioactive sea-water. No iodine was detected in the ssus threads whilst they were being formed within the foot, nor in any of e tissues responsible for the deposition of byssal material.

DISCUSSION

The experiments described here indicate that the main distribution of dine in bivalves exposed to the isotope, I^{131}, is on surfaces of the shell or dy in contact with the radioactive medium. Although there is a mechanism r the uptake of iodine into the tissues where it can subsequently be recogzed in the form of tyrosine-rich compounds labelled with the isotope orbman, Clements, and O'Brien, 1954), the iodine does not appear to be ncentrated in specific loci in the body. As also found by Fretter (1952) in periments with I^{131} on species of *Helix*, autoradiographs show that iodine scantily dispersed throughout the tissues, apart from deposits in the digese gland. Where iodine accumulates on the mantle, as occurs in *Anodonta*, is largely confined to the inner surface facing the mantle cavity.

The principal concentration of iodine occurs in association with the shell. orbman, Clements, and O'Brien (1954) considered that the shell and periacum of molluscs and the byssus threads of *Mytilus*, in common with horny uctures in other invertebrates (e.g. the exoskeleton of arthropods and the tae of polychaete annelids) can accumulate protein-bound iodine. However, hilst these authors clearly showed by radiochromatography the distribution iodine in the tissues of *Musculium* in the same chemical forms, monoiodotyrone and diiodotyrosine, as are found in the thyroid gland of vertebrates as ecursors of thyroxine, the present investigation found no evidence for the cretion of these substances into the shell. The accumulated iodine which have found in the shell and byssus of lamellibranchs must have been quired almost entirely from the surrounding medium and not by way of the sues of the body.

These deposits of iodine are located mainly on surfaces of the shell an byssus which have immediate access to the isotope, whereas the remainde of these structures show little or no tendency to concentrate the radioactiv material. Moreover, there is some evidence to suggest that the I^{131} associate with the shell has accumulated at least partially as a result of adsorption.

Only in that part of the shell secreted during or subsequent to treatmen with radioactive iodine could one possibly expect to find protein-boun iodine which had been deposited by the activity of the mantle. The mollusc used by Gorbman, Clements, and O'Brien (1954) were exposed to I^{131} fo only 24 to 48 h, during which time shell formation cannot have occurred t any appreciable extent. Even after the much longer exposure periods use in the present investigation, which ensured that the shell material was forme in the presence of radioiodine, the newly secreted parts of the shell gave wea or negative autoradiographs.

These features, coupled with the fact that iodine could not be detected i the regions of the mantle which secrete the shell, nor in the tissues formin the byssal threads in *Mytilus*, indicate that caution should be attached to th view that the isotope, I^{131}, can be utilized in shell and byssus secretion While it is possible that the failure to demonstrate iodine in the secretor tissues is due to there being a rapid turnover of very small amounts of th isotope in these regions of the mantle and foot (Jodfrey, 1953, for instance has shown that such a condition occurs in connexion with the calciu involved in shell deposition in the oyster), there appears at present to b insufficient evidence to show that I^{131} is actively incorporated in a protein bound form into the shell and byssus in the Lamellibranchia.

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REFERENCES

BEEDHAM, G. E., 1958*a*. Quart. J. micr. Sci., **99**, 181.
—— 1958*b*. Ibid. (in the press).
CAMERON, A. T., 1914. J. biol. Chem., **18**, 335.
—— 1915. Ibid., **23**, 1.
FRANCIS, G. E., MULLIGAN, W., and WORMALL, A., 1954. *Isotopic tracers*. London (Athlone
FRETTER, V., 1952. Quart. J. micr. Sci., **93**, 133.
GORBMAN, A., CLEMENTS, M., and O'BRIEN, R., 1954. J. exp. Zool., **127**, 75.
JODFREY, L. H., 1953. Biol. Bull., **104**, 398.
PEARSE, A. G. E., 1953. *Histochemistry*. London (Churchill).
POMEROY, L. R., and HASKIN, H. H., 1954. Biol. Bull., **107**, 123.
ROCHE, J., 1952. Experientia, **8**, 45.
ROCHE, J., RANSON, G., and EYSSERIC-LAFON, M., 1951. C.r. Soc. biol., **145**, 1474.
SCHOFFENIELS, E., 1951. Arch. internat. Phys., **58**, 467.
SIFFERT, R. S., 1948. Science, **108**, 445.
STEEDMAN, H. F., 1947. Quart. J. micr. Sci., **88**, 123.

The Gross Morphology of the Anterior Nervous System of *Nephtys*

By R. B. CLARK

(*From the Department of Zoology, University of Bristol*)

SUMMARY

The supra-oesophageal ganglion of *Nephtys* varies considerably in its size and gross orphology from one species to another. The ganglion is relatively larger in small ecies and then extends into the anterior body segments. It is epidermal and in con-ct with the cuticle in the prostomium, sub-epidermal in the anterior segments. In a umber of species a pair of conspicuous posterior lobes to the ganglion has been veloped. There is a tendency, particularly in the smaller worms, for the ganglion be bifurcated at its posterior, and sometimes also at its anterior margin. The sensory rves are as follows: tegumentary nerves, extremely variable in number and disposi-on, arising from the front of the ganglion; two pairs of antennary nerves arising mediately above the roots of the circum-oesophageal connectives, but generally stinct from them; and a pair of anterior optic 'nerves' supplying the paired, single-lled photoreceptors. The only other nerves arising from the ganglion are the nuchal rves which arise from it opposite the nuchal organs, whatever the position of the ain. Since the latter may barely protrude into the prostomium, these nerves emerge om the anterior part of the brain in some species, instead of near the posterior end the ganglion. The circum-oesophageal connectives are extremely long because the b-oesophageal ganglion lies in the fifth segment. In their passage through the lateral s of the mouth and along the sides of the gular membrane, they give off three pairs stomatogastric nerves and the segmental nerves of the first two segments. The third d fourth segmental nerves arise from the front of the sub-oesophageal ganglion, ose of the fifth segment from the back of the ganglion. The stomatogastric nervous stem is also described. The entire nervous system shows little specialization com-red with that of most other errant polychaetes.

INTRODUCTION

THE supra-oesophageal ganglion of *Nephtys* varies considerably from one species to another. This is surprising in a family of polychaetes hich otherwise shows great uniformity of structure, and the variability is t restricted to the gross anatomy of the brain; there are considerable and portant differences in its fine structure between species. Some of the reasons r this variation have already been discussed (Clark, 1957) and I propose give an account of the fine structure of the ganglion in a later paper. At esent I am concerned with the gross anatomy of the supra-oesophageal nglion and its variation in the Nephtyidae. The variability does not extend other parts of the nervous system, but as existing accounts of these parts e unsatisfactory in several respects, a description of them is also included.

The literature includes seven accounts of the anatomy of the nervous system *Nephtys*, by Delle Chiaje (1825), Quatrefages (1850), Ehlers (1864–8), laparède (1868), Pruvot (1885), Schack (1886), and de Saint-Joseph (1894). ll deal with *N. caeca*, *N. cirrosa*, and *N. hombergi*. Of these accounts, that

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by Pruvot is the most ambitious. Mistakes occur in all these studies and i none of them is a comparative account of the nervous system attempted Even where there are gross differences in the morphology of the nervou system between these three species, the authors do not agree in their observation, far less in their interpretation. Quatrefages described a chain of sever ganglia anterior to the supra-oesophageal ganglion and lying between th circum-oesophageal connectives in the prostomium. Later authors correcte this error and concluded that Quatrefages had mistaken a mass of connectiv tissue or muscle for ganglia. In fact the mass is glandular. There was also difference of opinion between Quatrefages and de Saint-Joseph, on the on hand, and Ehlers and Schack, on the other, as to whether or not posterio lobes were attached to the supra-oesophageal ganglion of *N. caeca*. Thi apparently arose from a misidentification of the worms, for the lobes occu in *N. caeca*, but not in *N. hombergi* and a number of other species. The question has been resolved in favour of the German authors (Clark, 1955). Pruvo and de Saint-Joseph concluded that the sub-oesophageal ganglion lay in th fourth segment (in fact it is in the fifth) and as a result incorrectly describe the innervation of the first four segments. Thus, although there is much tha is correct and valuable in them, these seven accounts are too full of discrepancies and error to be reliable or to inspire confidence.

MATERIAL AND METHODS

The species of *Nephtys* which have been used in this study are: *N. bucer* Ehlers, *N. caeca* (Fabricius), *N. caecoides* Hartman, *N. californiensis* Hartman *N. cirrosa* Ehlers, *N. cornuta* Berkeley and Berkeley, *N. ferruginea* Hartman *N. glabra* Hartman, *N. hombergi* Audouin and Edwards, *N. incisa* Malmgren *N. longosetosa* Oersted, *N. magellanica* Hartman, *N. parva* Clark and Jones *N. picta* Ehlers, *N. punctata* Hartman, *N. rickettsi* Hartman, and *N. squamos* Hartman. The structure of the nervous system has been elucidated by dissection and by the examination of serial frontal, sagittal, and transverse section of material fixed in Bouin's or Helly's fluids, and stained with paraldehyd fuchsin, Mallory triple stain, Azan, and Heidenhain's iron haematoxylin.

THE EPIDERMAL NATURE OF THE NERVOUS SYSTEM

The nervous system of all species of *Nephtys* is to varying degrees epidermal that is to say, its embryological epidermal origin is manifest in the adult and as in a number of the more primitive polychaetes, parts of the nervous system are not clearly separated from the epidermis. This is most evident in th supra-oesophageal ganglion. It is bounded laterally and ventrally by a connective-tissue sheath which is continuous with the epidermal basement membrane, and the dorsal surface of the ganglion is in contact with the prostomia cuticle. In most species part of the ganglion extends into the anterior bod segments, and in them the ganglion is suspended beneath the epidermis an is completely invested by its sheath (fig. 10, p. 217); only that part of th ganglion which lies in the prostomium is in contact with the cuticle (fig. 8, A

215). Probably the whole ganglion was originally prostomial and epidermal, t in the course of evolution of these worms it has tended to extend into the terior segments as a sac beneath the epidermis (Clark, 1957, fig. 3). The idermal cells of the prostomial wall stop abruptly at the sides of the gang- n, and the cells in the dorsal part of the ganglion which replace them pear to be modified neuroglial cells. Goblet or coiled mucus-cells, which e scattered in the prostomial epidermis, do not occur in the ganglion.

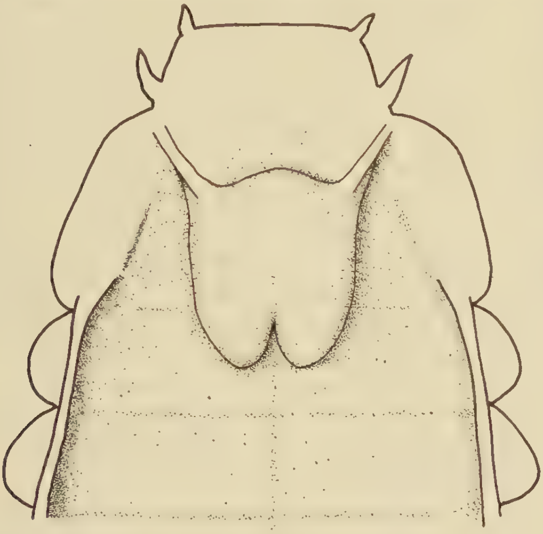


FIG. 1. Supra-oesophageal ganglion of *N. incisa*, ventral view.

Other parts of the nervous system, namely the ventral nerve-cord and sub- sophageal ganglion, the segmental nerves, the stomatogastric nervous stem, and the prostomial nerves, are all intimately connected with the idermis, but never to the extent of replacing it as the supra-oesophageal nglion does. All these structures lie in the sub-epidermal basement mem- ane and are separated from the cuticle by a layer of quite typical epidermal lls which differ in no way from those in other parts of the body-wall.

THE SUPRA-OESOPHAGEAL GANGLION

The supra-oesophageal ganglion of *N. incisa* (fig. 1) is subject to the fewest f the modifications discussed below and it will serve best for a description f the architecture of that organ. It lies in the posterior half of the prostomium d is as long as broad, with a slight bifucation in its posterior margin. Only e posterior tips of it project into the first segment, and from the foregoing scussion it will be clear that almost the entire ganglion is in contact with the ostomial cuticle. Only the extreme posterior tips are suspended beneath e epidermis. There is a groove in the ventral surface of the ganglion and the rsal blood-vessel runs along it, bifurcating in the middle part of the gang- n, the two branches diverging and passing ventrally and having no further contact with the brain (Clark, 1956*a*). The sheath investing the ganglion i thickened in this ventral groove and in the anterior part of it is drawn ou into a process for the attachment of the prostomial muscles (as in fig. 8, A, B) A series of thick fibres traverses the ganglion in the mid-line and runs dorso ventrally from the cuticle to the sheath investing the brain (as in fig. 8, B) They correspond with the insertion of the muscles on the other side of th sheath and presumably prevent too great distortion o the ganglion when the muscles are contracted. Th fibres do not form a continuous sheet bisecting th brain, but are arranged in several groups with space between to permit neural inter-connexions.

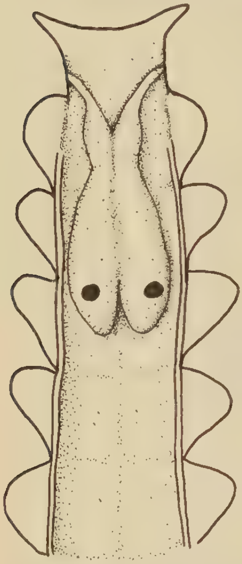


Fig. 2. Supra-oesophageal ganglion of *N. cornuta*, ventral view.

In accordance with von Haller's rule, the relativ volume of the supra-oesophageal ganglion of *Nephty* varies inversely with the size of the species (Clark 1957). In all species the ganglion is of approximatel the same width as the prostomium and differences i the relative volume of the brain are accounted for b an increase in its length. In small species the relativel long ganglion cannot be accommodated entirely withi the prostomium and it extends backwards into th anterior body segments, so much so that in the two ver small species *N. cornuta* and *N. parva* it extends int the third body segment (figs. 2, 3).

In nearly all species, whatever the relative size of th ganglion, it tends to lie mainly in the first body segmen rather than in the prostomium. Thus, it is unusual t find more than a small part of the ganglion in th prostomium itself and, as a rule, most of it, unlike tha of *N. incisa*, is sub-epidermal. The fact that not more than a small part of th ganglion lies in the prostomium has some influence on the relationship between the brain and the dorsal blood-vessel and the prostomial muscles, sinc these bear a fixed topographical relationship to the prostomium rather than t the supra-oesophageal ganglion. In those species in which the ganglion i mainly in the prostomium, the prostomial muscles are attached to the anterio half of the brain, but if the ganglion does not protrude far into the prostomium, the muscles are, of course, attached only to the anterior margin o it. In the same way, the dorsal blood-vessel which bifurcates half-way along or even near the posterior part of the ganglion of those species with a largel prostomial brain, runs undivided along the whole length of the ganglion i species in which it occupies a more posterior position.

A source of variation in the morphology of the ganglion, quite unrelate to the size of the worm or its brain, is the presence of posterior lobes in som species (figs. 3, 4, 6). Except in *N. cirrosa*, these structures are filled wit mucus-cells, the long necks of which run in a tract on each side of the ganglio and open to the exterior in the lateral walls of the prostomium (Clark, 1955) he entire mucus-gland system is enclosed within the sheath which invests e ganglion and is separated from the nervous tissue by a barrier of neuroglial lls. The lobes may be very long and occupy a much greater volume than the nglion itself. Thus in *N. caecoides* they extend from the posterior margin the supra-oesophageal ganglion in the first segment, to the fifteenth seg- ent; in *N. caeca* only to the fifth segment. Posterior lobes are missing from e brains of the following species; *N. cornuta*, *N. hombergi*, *N. incisa*, *N.* *icta*, and *N. squamosa*, although the posterior margin of the ganglion is often ightly bifurcated, giving the spurious impression of small posterior lobes. /. *cirrosa* also has posterior lobes, though they are of a somewhat different ature from those in other species. In them, the mucus-cells appear to have een modified and more or less incorporated within the nervous system lark, 1956*b*).

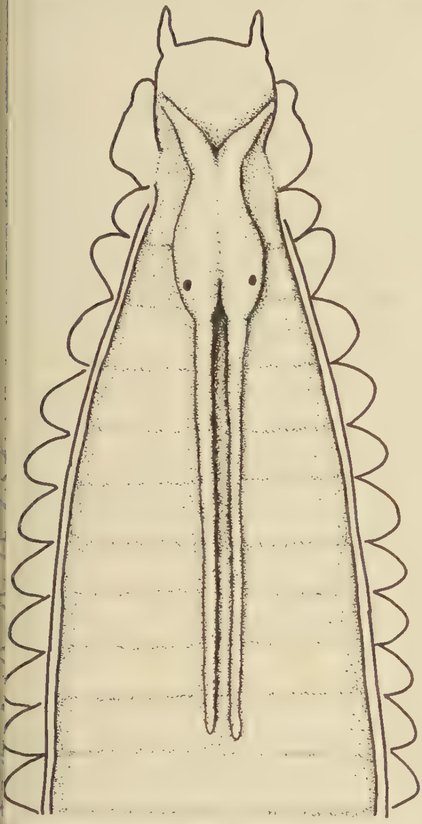


FIG. 3. Supra-oesophageal ganglion of *N. parva*, ventral view.

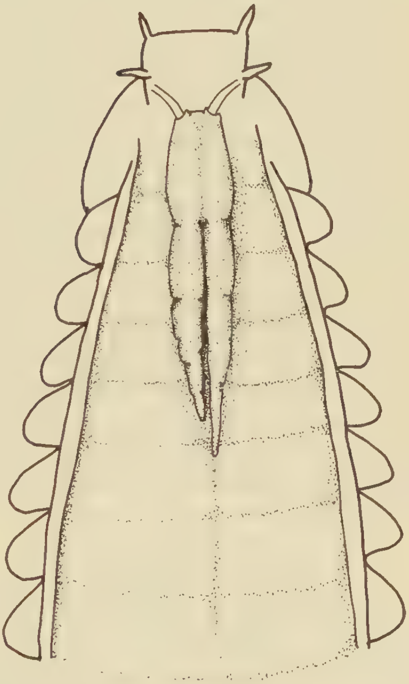


FIG. 4. Supra-oesophageal ganglion of *N. ferruginea*, ventral view.

There is considerable interspecific variation in the shape of the ganglion, articularly among the small-sized species, quite apart from differences in elative size and the occurrence or degree of development of the posterior lobes. In worms larger than *N. incisa*, the ganglion is shorter than broad roughly trapezoidal, and generally with only a slight bifurcation in its posterior margin and none in the anterior (e.g. *N. caeca*, *N. caecoides*, *N. californiensis* *N. punctata*, *N. rickettsi*, all of which possess posterior lobes (fig. 6; see Clark, 1955, fig. 1)). In *N. hombergi* (fig. 7), a species which lacks posterior lobes, the posterior bifurcation is more obvious. In the small worms, however the ganglion is elongated, extending into the anterior body segments and therefore largely sub-epidermal, and it has a very different appearance because of a tendency for it to be deeply bifurcated. Indeed, in the two smallest species, *N. cornuta* and *N. parva* (figs. 2, 3), the ganglion is deeply bifurcated at both anterior and posterior margins and has a pronounced ventral longitudinal groove in it so that it appears to be almost a double structure. The supra-oesophageal ganglion of *N. ferruginea* (fig. 4) has some peculiarities not shown by that of other species. It is barrel-shaped and much longer than that of *N. picta*, a species of comparable size. This is because the lateral tract of ducts from the cells in the posterior lobes of *N. ferruginea* is extremely well developed and occupies much of the cross-sectional area of the ganglion (fig. 8, B), while *N. picta* lacks posterior lobes and the lateral tracts of ducts. The volume of the truly ganglionic material (nerve-cells and neuroglia) is the same in both species, so the nervous part of the ganglion of *N. ferruginea* is elongated to compensate for its reduced cross-sectional area. A further peculiarity of the ganglion of *N. ferruginea* is that the circum-oesophageal connectives emerge from cup-shaped depressions at the antero-lateral corners of the ganglion and have a ventral flange covering the point of emergence of the connectives. This ornamentation of the anterior end of the ganglion does not occur in any other species, and I am at a loss to explain its occurrence in this one.

THE CIRCUM-OESOPHAGEAL CONNECTIVES

The circum-oesophageal connectives leave the supra-oesophageal ganglion at its antero-lateral corners, or, in the case of some small species, anteriorly. Macroscopically, they appear to emerge as single structures, not double as they are shown in Pruvot's (1885) illustration, though, in fact, they have a double origin in the supra-oesophageal ganglion and the two tracts of fibres retain their identity along the whole length of the connectives and into the sub-oesophageal ganglion. A short distance in front of the supra-oesophageal ganglion the connectives turn and run ventrally to the ventral surface of the prostomium, meeting it at the point where the lateral lips of the mouth are fused with it. The connectives then run posteriorly in the edges of the lips, giving off three stomatogastric nerves and the segmental nerves of the first segment. In the third segment the connectives leave the lateral lips and run in the ventral body-wall on each side of the gular membrane, converging on the sub-oesophageal ganglion in the fifth segment. In their passage through segments II, III, and IV the connectives are accompanied by the circum-oral blood-vessels, and, like them, are not subjected to tension when the proboscis

everted, because the lateral lips are thrust aside and the gular membrane is
retched as the large pharynx passes through these segments; the body-wall
which the blood-vessels and nerves run is unaffected (Clark, 1956*a*).

THE SUB-OESOPHAGEAL GANGLION

The ganglia of the ventral nerve-chain of *Nephtys* are merely slight swell-
gs on the nerve-cord. They are intersegmental in position, the greater part
each ganglion lying in the anterior part of the segment it principally
nervates. The sub-oesophageal ganglion is a little larger than the other
terior ganglia, but otherwise not distinguished from them. It lies at the
sterior end of the gular membrane, in the fifth segment, and the circum-
sophageal connectives are therefore a good deal longer than is usual in
lychaetes. The connectives enter the ganglion at its antero-lateral corners.
mediately below and behind the point of entry of the connectives, the
gmental nerves of the third and fourth segments emerge from the ganglion.
he sub-oesophageal ganglion, like the connectives and the segmental nerves,
s in the sub-epidermal basement membrane, which is appropriately ex-
nded to accommodate the bulky ventral nerve-cord.

THE NERVES OF THE SUPRA-OESOPHAGEAL GANGLION

The sensory apparatus of the prostomium of *Nephtys* is somewhat reduced,
ough apart from the absence of palps, it is comparable to that of most other
rant polychaetes in that there are two pairs of antennae, two pairs of photo-
ceptors, and a pair of nuchal organs. In addition, there are numerous
ticular sensory hairs, mainly on the dorsal surface of the prostomium.
he sensory nerves of all these structures enter the supra-oesophageal
nglion.

The tegumentary nerves. The single-celled receptors of the dorsal surface
the prostomium and, in part, those in the antennae, are innervated by a
ries of tegumentary nerves which arise from the extreme dorsal part of the
pra-oesophageal ganglion and emerge from its anterior margin. These
rves are extremely variable in their arrangement, not only from one species
another, but also between individuals of the same species, and even in a
ngle worm the arrangement of these nerves may differ in right and left
lves of the prostomium. As many as eight tegumentary nerves may arise
om the ganglion, or there may be only two. In the former case one nerve on
ch side is larger than the others and innervates the greater part of the
ostomium. Often there are anastomoses between the branches of the nerves.
me examples of the arrangement of the tegumentary nerves in different
ecies are given in figs. 5, 6, and 7. Fig. 6 shows a specimen of *N. caeca*
which the innervation of the right and left sides of the prostomium is
fferent.

The antennal nerves. The first antennae lie at the antero-lateral corners of
e prostomium, the second about half-way along its sides. Two pairs of

antennal nerves arise in the dorsal part of the supra-oesophageal ganglion and emerge immediately above the roots of the circum-oesophageal connectives. These nerves have a separate origin in the ganglion and, in mos species, remain quite distinct from the connectives. The nerves run in th lateral walls of the prostomium to their respective antennae, where the

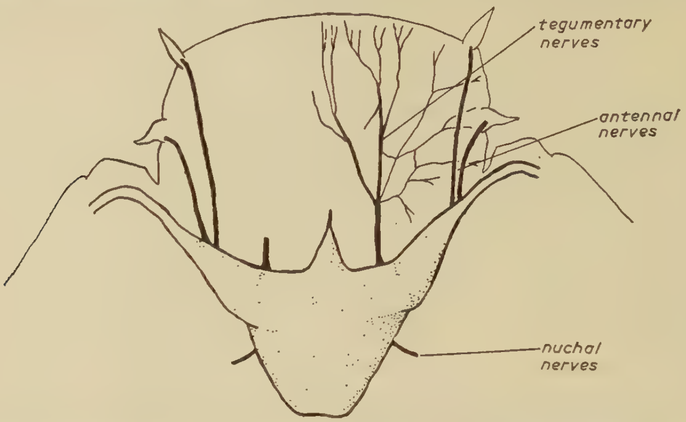


FIG. 5. Supra-oesophageal ganglion and cephalic nerves of *N. bucera*, ventral view. Only th root of the tegumentary nerve of the left side is shown.

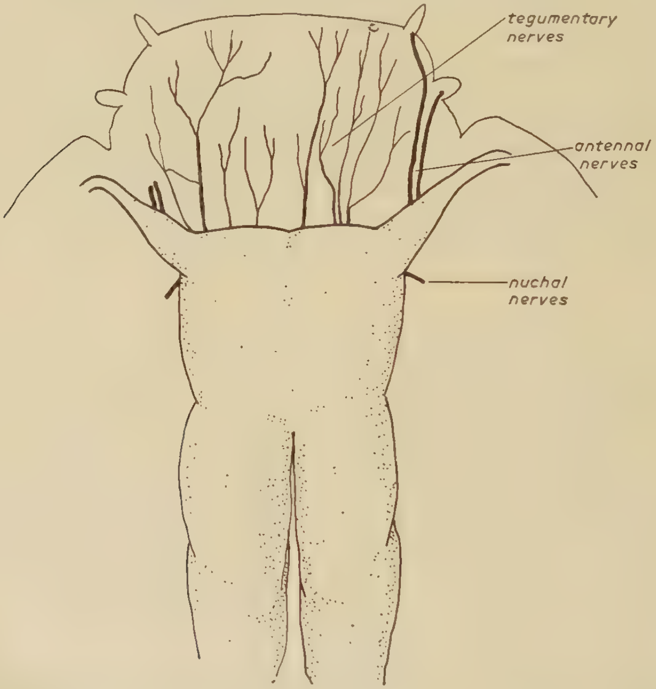


FIG. 6. Supra-oesophageal ganglion and cephalic nerves of *N. caeca*, ventral view. Only th roots of the antennary nerves of the left side are shown.

eak up into a number of fine branches. In *N. hombergi* (fig. 7) the second tennal nerve is in a slightly more lateral position than it is in other species d it runs in the connective for a short distance, emerging from it near the se of its appropriate antenna. It is also unusual in that it innervates a small ea of the prostomial wall as well as the antennal sense organs.

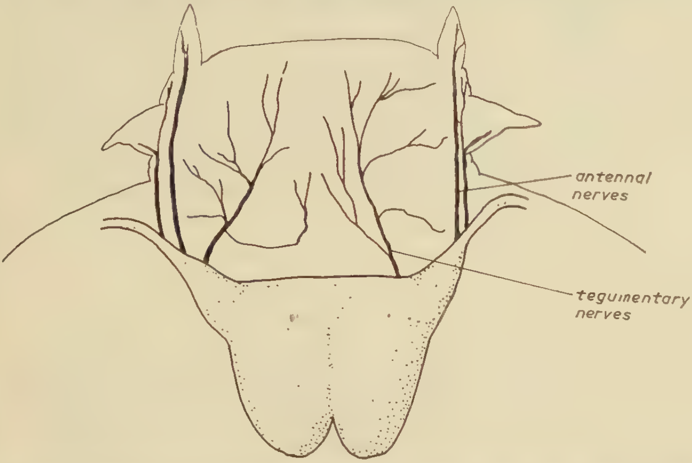


FIG. 7. Supra-oesophageal ganglion and cephalic nerves of *N. hombergi*, ventral view.

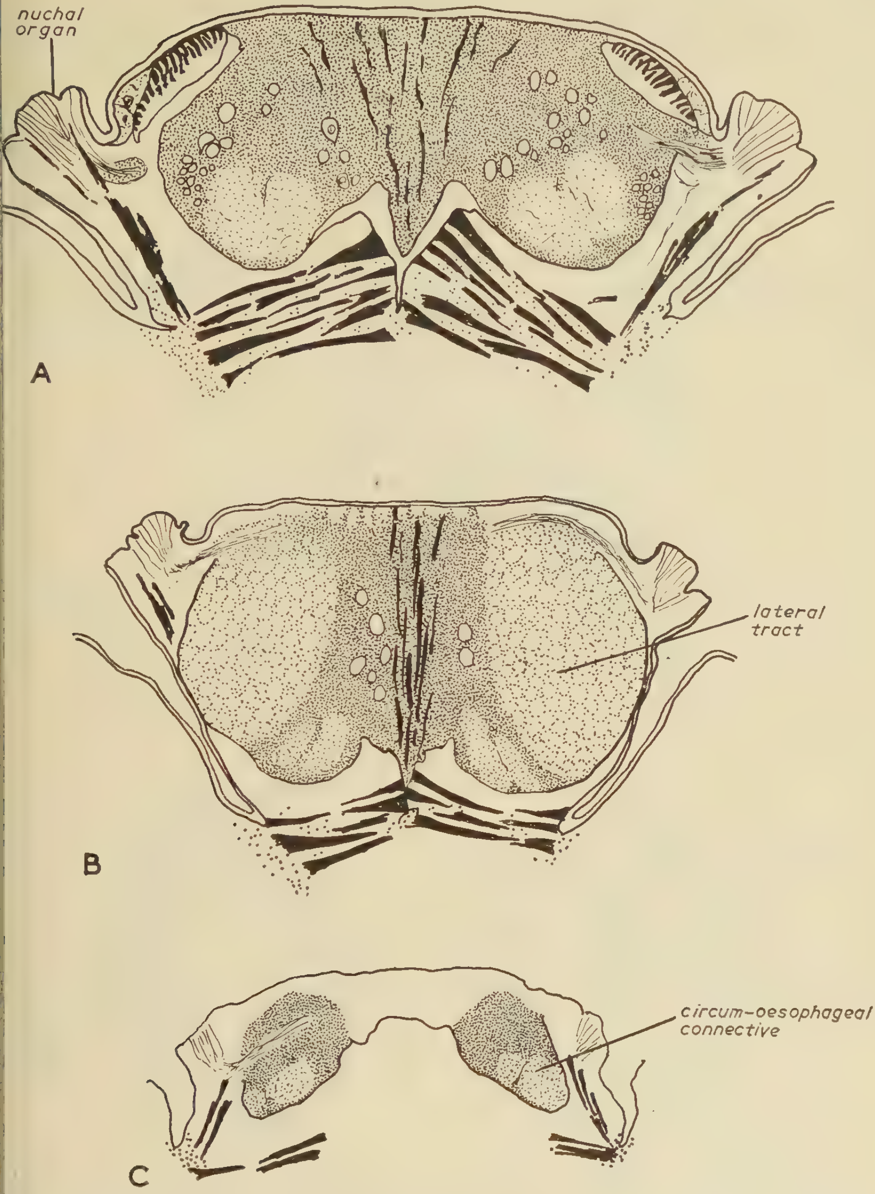
The optic nerves. The posterior photoreceptors with their pigment cups e embedded in the supra-oesophageal ganglion and there are no external rves. These receptors have not been identified (though they are probably esent) in *N. incisa* or *N. squamosa*, but otherwise they have been found in species and always have the same structure. The anterior photoreceptors, nsisting of a pair of sensory cells on each side, without pigment cups, lie just terior to the roots of the circum-oesophageal connectives in the epidermis the dorsal surface of the prostomium. Axons from these receptors enter e ganglion a little lateral to the connectives. The anterior receptors bear a nstant relationship to the brain and are never far from its anterior margin, that they may lie at the level of the nuchal organs (in *N. picta*) or farther rwards (as in *N. caeca*), depending upon the position of the ganglion in the ostomium. Anterior receptors appear to be missing from *N. punctata* and . *rickettsi*. The structure and functioning of these photoreceptors have been scribed in more detail elsewhere (Clark, 1956*c*).

The nuchal nerves. The paired nuchal organs of *Nephtys* are small, spherical uctures between 10 and 50 μ in diameter, depending upon the size of the ecies, and lying at the postero-lateral margins of the prostomium. They are ersible and are generally found everted in preserved specimens. Each is ovided with a retractor muscle which is inserted in the base of the pro- omial wall where it joins the wall of the first segment, and where the diagonal ostomial muscles are also inserted. In *N. incisa* the nuchal nerve consists a small bundle of fibres which crosses the short intervening distance between the organ and the dorso-lateral part of the ganglion. In this species in which the ganglion is almost entirely prostomial, the nuchal organs ar almost at the same level as the nuchal centres in the ganglion. For the sake o comparison with other species, it should be noted that at this level the dorsa blood-vessel is attached to the ventral surface of the brain, and the centra neuropile in the ganglion forms a single, compact mass. In some species th ganglion is elongated and in most it is shifted backwards into the anterio body segments; in either case the posterior part of the ganglion, in which the nuchal centres are situated, lies some distance behind the nuchal organs In fact, *N. incisa* is the only species in which the nuchal centres and organ are close together. It might have been expected that the posterior shifting o the ganglion would have resulted in an elongation of the nuchal nerve. Thi is not so. In all species of *Nephtys* the nuchal nerve emerges from the brai at the level of the nuchal organs, even though this may result in the nerv leaving the ganglion at its anterior edge. Fig. 8, A–C shows cross-sections o the prostomium of *N. cirrosa*, *N. ferruginea*, and *N. cornuta* at the level o the nuchal organs. In the first two species the section passes anterior to th dorsal blood-vessel and through the region where the prostomial muscle are attached to the base of the ganglion. In both, the neuropile is divided u to form the roots of the circum-oesophageal connectives and in *N. ferrugine* these nerve-bundles are at the sides of the ganglion immediately before the emerge from the brain. In *N. cornuta* a section through the nuchal organs i actually anterior to the ganglion and the nerves enter the neuroglial tissu that lies on top of the roots of the connectives in this species. In a specie such as *N. ferruginea*, which possesses posterior lobes, the nuchal organs li at the posterior ends of the lateral organs, where the mucus-cells containe in the posterior lobes open to the exterior. The nuchal nerves enter th ganglion dorsal to the lateral tracts of ducts running from the posterior lobe to the lateral organs.

The Stomatogastric Nervous System

The proboscis of *Nephtys* consists of a thin-walled buccal region and very muscular pharynx. When the proboscis is everted, the buccal region form a sheath around the protruded pharynx. The buccal sheath is attached t the body-wall beneath the prostomium, to the lateral lips of the mouth, and ventrally, to the anterior end of the folded gular membrane. At the anterio end of the pharynx, that is at the tip of the everted proboscis, there are 2 large papillae arranged in two vertical rows of 11 each, which are provide with muscles so that they may be drawn back. The sub-terminal papillae which are used as diagnostic features in nephtyid taxonomy, are merel evaginations of the buccal sheath and are not movable. The opening of th pharynx is a vertical slit which is bounded top and bottom by two strong ligaments (fig. 9, C). Although the opening is a dorso-ventral slit, the cavity o the pharynx is cross-shaped (fig. 9, D).

Three stomatogastric nerves arise from the circum-oesophageal connective



G. 8. A, transverse section through the prostomium of *N. cirrosa* at the level of the nuchal gans, showing the nuchal nerve entering the ganglion. B, a similar section through the ostomium of *N. ferruginea*. The nuchal nerves run to the ganglion over the top of the eral tracts of ducts from the posterior lobes. C, a similar section through the prostomium *N. cornuta*. The nuchal nerves enter the neuroglia, which lies above the roots of the cir-m-oesophageal connectives and anterior to the ganglion proper. Dense stipple, neuroglia; light stipple, neuropile and circum-oesophageal connectives.

on each side in their passage through the lateral lips of the mouth (figs. 9, A; 10). The third, most ventral, stomatogastric nerve gives off a fine side branch,

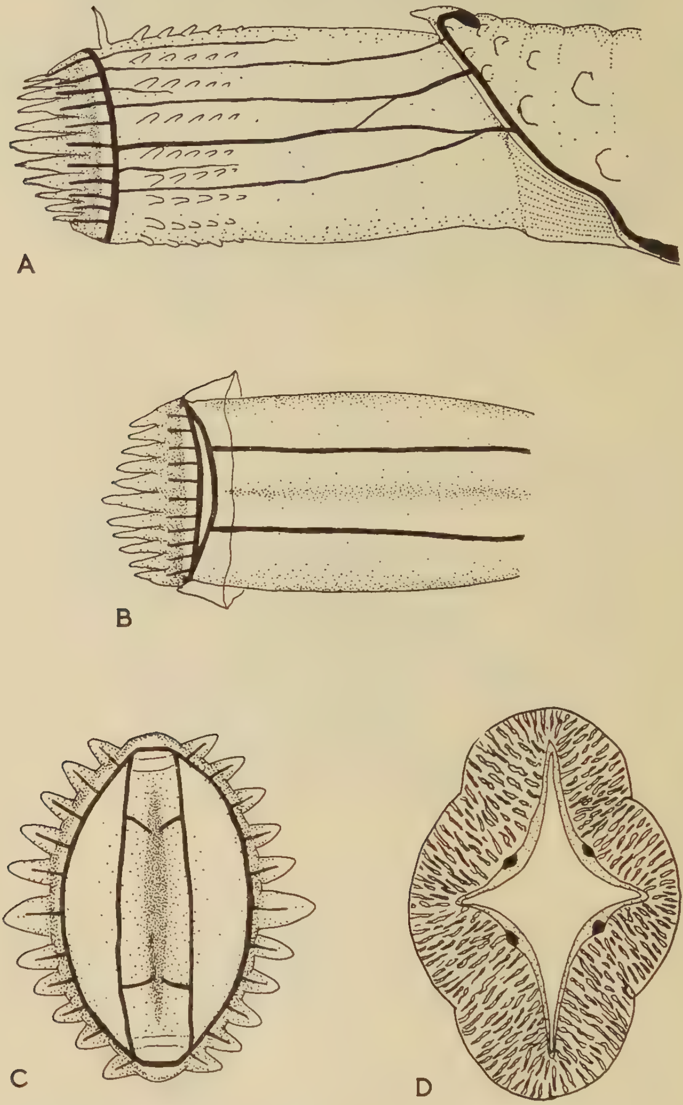
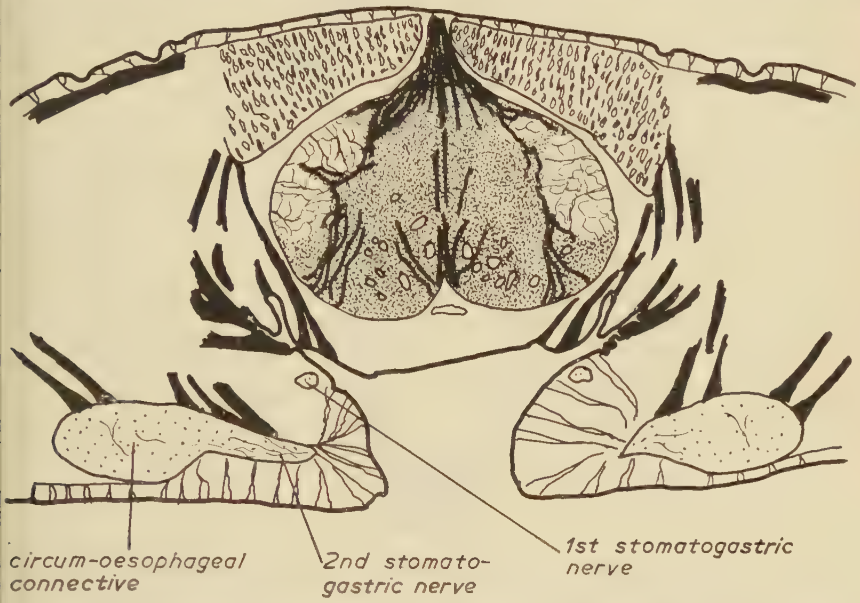


Fig. 9. A, buccal part of the stomatogastric nervous system of *N. hombergi*. B, pharyngea part of the stomatogastric nervous system of *N. hombergi*. C, oral view of the proboscis o *N. bucera* to show the double stomatogastric nerve-ring at the anterior end of the pharynx D, transverse section through the pharynx of *N. glabra* to show the position of the fou pharyngeal stomatogastric nerves.

and these four pairs of nerves run along the buccal sheath to a nerve-ring a the base of the terminal proboscidal papillae. The second and third stomato gastric nerves are the most substantial, the first and fourth are fine. There i arge anastomosis between the second and third nerves, and, in most species, ine one between the first and second. A number of fine nerves arise from e terminal ring-nerve and run back along the buccal sheath, though they not form a connexion with the circum-oesophageal connectives. Twenty-o nerves arise from the ring and enter the terminal papillae. In addition the nerve-ring at the base of the papillae, there is a second ring near the terior end of the pharynx (fig. 9, B, C). This is joined to the terminal ring each side, dorsally and ventrally, so that only a single nerve runs above d below the ligaments at the top and bottom of the pharyngeal opening. ur nerves arise from the pharyngeal ring and run back along the four main uscle-blocks composing the pharynx. They lie at the base of the epithelium ing the pharynx (fig. 9, D).



. 10. Transverse section through the first segment of *N. californiensis* to show the origin the second stomatogastric nerve from the left circum-oesophageal connective. The first ir of stomatogastric nerves lies in a more dorsal and internal position in the lateral lips.

The Segmental Nervous System

Since the sub-oesophageal ganglion lies in the fifth segment, some modi-ation of the segmental nerves of the first four segments must be expected. e most conspicuous segmental nerve is the parapodial nerve, which leaves e posterior part of the ventral ganglion in typical segments and runs to a dial ganglion at the base of the appropriate parapodium. From this gang-n a series of nerves innervate the noto- and neuropodia and the rest of e parapodium. The parapodia of the fifth segment are innervated in the ual way by nerves arising from the posterior part of the sub-oesophageal ganglion (fig. 11). The parapodial nerves of the third and fourth segment arise from the anterior part of the sub-oesophageal ganglion immediately behind the circum-oesophageal connectives. In both cases, the segmental nerve follow the connectives and branch off at the appropriate level to supply th parapodia and body-wall of their respective segments. These nerves clearl arise in the sub-oesophageal ganglion, although they become closely associate with the circum-oesophageal connectives. The segmental nerves of the firs two segments, however, arise from the connectives themselves and have n obvious connexion with the sub-oesophageal ganglion, although presumabl their innervation is ultimately from that source. Two widely spaced nerve arise from the circum-oesophageal connectives in their passage through th lateral lips and converge on the podial ganglion at the base of the first segmen The parapodial nerve of the second segment is single and arises from th connectives at the point where they leave the lateral lips and enter the ventra body-wall, i.e. at the anterior end of the gular membrane. This agrees wit the description given by Pruvot (1885), except that he omitted the segmenta nerves of the second segment and concluded that the sub-oesophageal ganglion lay in the fourth instead of the fifth segment.

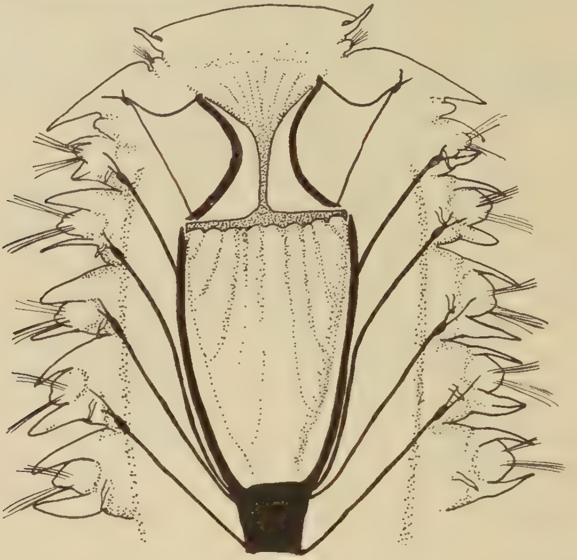


FIG. 11. Ventral view of the anterior nervous system of *N. bucera* to show the path of th circum-oesophageal connectives and the innervation of the first five segments.

DISCUSSION

Although the general pattern of the nervous system of *Nephtys* resemble that of other errant polychaetes, it is useless to discuss homologies of th nerves at this stage because differences in the point of emergence of nerve from the central nervous system do not necessarily indicate differences in th rangement of the ganglionic centres from which they come. The variable osition of the second antennal nerves and, still more, of the nuchal nerves *Nephtys* are cases in point; they do not reflect a variation in the disposition the antennal or nuchal nuclei within the brain.

The supra-oesophageal ganglion appears to be much less specialized than hat of eunicids and aphroditids, for example, in which the development of n elaborate prostomial sensory system is associated with large and compliated supra-oesophageal ganglia (Hanström, 1928). Superficially, the nervous ystem of *Nephtys* bears a closer relationship with that of the nereids or hyllodocids, though in both these families there is a more elaborate protomial sensory apparatus than in *Nephtys*. In these two families, two or ore of the anterior segments are fused to form a peristomium and their entral ganglia are also fused, and the segmental nerves come into a close ssociation with each other. A rather similar disturbance of the nervous ystem of the anterior segments occurs in *Nephtys*, though for different easons. The proboscis is much greater in diameter than the anterior segments rough which it passes. The first four segments are particularly small and re provided with a ventral gusset in the form of a folded gular membrane, hich is stretched when the proboscis is everted. The sub-oesophageal anglion lies at the posterior margin of the gular membrane, and the circumesophageal connectives, which run along the sides of the membrane, are onsequently remarkably long. As we have seen, the innervation of the third, urth, and fifth segments, and possibly of the first and second also, is from e sub-oesophageal ganglion which, as in the nereids and phyllodocids, ust be a composite structure formed from several ventral ganglia. Unlike ese families, however, the segmental nerves of *Nephtys* are not affected by ese modifications and do not come into any special, close relationship with ach other.

The chief sources of variation in the gross structure of the anterior nervous ystem are the occurrence of posterior lobes and the great size range of species f this genus. The extreme variability of the tegumentary nerves of the rostomium is a reflection of the lack of discrete sense organs and is unimporant. The chief characteristic of the nervous system of these worms is its ck of specialization coupled with a reduced or primitive sensory system.

I am indebted to Dr. Marion H. Pettibone for collections of *N. bucera* ade for me in Maine and New Hampshire.

REFERENCES

LAPARÈDE, E., 1868. 'Les Annélides chétopodes du Golfe de Naples.' Mem. Soc. phys. Hist. nat. Geneva, **19**, 313.

LARK, R. B., 1955. 'The posterior lobes of the brain of *Nephtys* and the mucus-cells of the prostomium.' Quart. J. micr. Sci., **96**, 545.

— 1956*a*. 'The blood vascular system of *Nephtys* (Annelida, Polychaeta).' Ibid., **97**, 235.

— 1956*b*. 'On the origin of neurosecretory cells.' Ann. Sci. nat. Zool., **18**, 199.

Clark, R. B., 1956*c*. 'The eyes and the photonegative behaviour of *Nephtys* (Annelida Polychaeta).' J .exp. Biol., **33**, 461.

—— 1957. 'The influence of size on the structure of the brain of *Nephtys*.' Zool. Jahrb. Abt Physiol., **67**, 261.

Delle Chiaje, S., 1825. *Memorie sulla Storia e Notomia degli Animali senza Vertebre de Regno di Napoli*, **2**. Naples.

Ehlers, E., 1864–8. *Die Borstenwürmer*. Leipzig.

Hanström, B., 1928. *Vergleichende Anatomie des Nervensystems der Wirbellösen Tiere*. Berlin (Springer).

Pruvot, G., 1885. 'Recherches anatomiques et morphologiques sur le système nerveux de Annélides polychètes.' Arch. Zool. exp. gén., **3**, 211,

Quatrefages, A. de, 1850. 'Études sur les types inférieurs de l'embranchement des Annélides. Ann. Sci. nat. Zool., **14**, 329.

Saint-Joseph, A. de, 1894. 'Les Annélides chétopodes des côtes de Dinard.' Ann. Sci. nat Zool., **17**, 1.

Schack, F., 1886. *Anatomisch-histologische Untersuchung von* Nephthys coaeca *Fabricius* Kiel (Zool. Inst.).

Some Observations on the Glycogen of the Wool Follicle

By M. L. RYDER

(*From the Wool Industries Research Association, Torridon, Headingley, Leeds* 6)

With one plate (fig. 1)

Summary

The distribution of glycogen in the wool follicle is described; its presence, until cently unsuspected, has been demonstrated in the unkeratinized part of the fibre. he deposition of glycogen in the follicle has been investigated by injecting mice with ucose labelled with carbon-14.

The distribution of acid and alkaline phosphatases is described; the former appears be concentrated in the unkeratinized part of the fibre and the latter in the blood-ssels of the follicle.

Introduction

T has long been known that there is glycogen in the outer sheath of the follicle and in the fibre medulla. Only recently, however, has glycogen een demonstrated in the rest of the unkeratinized (human) hair fibre (Mon-gna, Chase, and Lobitz, 1952). Ryder (1957*a*) reviewed previous work on the ycogen of the follicle and gave some preliminary observations on the sheep.

The distribution of alkaline phosphatase in the follicle was studied by hnson, Butcher, and Bevelander (1945) in the rat, by Hardy (1952) in the ouse, and in man by Braun-Falco (1957), who in addition reviewed previous ork and studied acid phosphatase, which has also been studied by Moretti nd Mescon (1956) in man. Ellis, Gillespie, and Lindley (1950) made some rief observations on the acid and alkaline phosphatase in wool follicles.

Material and Methods

For glycogen, the skin of Masham and Devon longwool sheep was fixed in uffered 5% formalin (Pearse, 1953). It was sectioned at a thickness of 8 μ orizontally and vertically to the skin surface. The method for glycogen of otchkiss (1948) and the modification by Montagna, Chase, and Hamilton 951) were used, as well as Best's carmine. In Montagna's modification ronger oxidation is applied by increasing the time of exposure to periodic cid from 5 to 10 min and the temperature to 37° C. Control slides containing lternate sections were placed in 1% diastase solution for 1 h before this reatment.

In the first experiment with glucose labelled with carbon-14, five mice were njected with about 30 μc of the compound each, and killed 1 h, 8 h, 24 h, days, and 10 days afterwards. The zone of active growth was indicated by yeing the coats of the mice; but plucking was later found to be a better way f obtaining active follicles (Chase, Rauch, and Smith 1951), and this was used in the second experiment. In this four mice were injected with abou 37 μc each, about 13 days after plucking, and killed 1 h, 8 h, 24 h, and 3 day afterwards. Samples of skin were sectioned horizontally and vertically at 5 and, after staining with Montagna's modification, Kodak autoradiographi film (A.R. 10) was applied to the sections by the stripping technique o Doniach and Pelc (1950). Exposures of 60, 84, and 112 days were given. In th first experiment frozen sections 50 μ thick were cut of the vibrissae and Koda A.R. 50 film applied (fig. 1, B, opposite p. 224).

Gomori's lead-phosphate method for acid phosphatase, and his calcium cobalt method for alkaline phosphatase were used, as well as the azo dy coupling methods (all from Pearse, 1953) with samples of Romney sheep skin In addition for alkaline phosphatase the method of Manheimer and Seligma (1948) was used with sodium α-naphthyl phosphate as substrate.

No success was obtained with the method for phosphorylase of Takeuch and Kuriaki (1955), not even with liver and muscle, although several batche of insulin were tried, whereas Braun-Falco (1956) obtained a reaction in th outer sheath of human hair follicles.

RESULTS

Stains showing glycogen

With Best's carmine and Hotchkiss's methods, the largest amount of glycogen in the follicle was in the cytoplasm of the outer sheath cells. There were however, variations between follicles, and with Best's carmine some follicle appeared to have hardly any glycogen. Animals receiving a poor diet showe no reduction in the amount of glycogen in the follicle. Montagna's modification always showed more glycogen than the other methods (fig. 1, A).

The greatest concentration of glycogen in the outer sheath was in the lower half of the follicle, above the neck of the bulb. Montagna's modification showed glycogen to be present in smaller quantities as high as the skin surface The pink Best's carmine reaction was often diffuse throughout the cytoplasm, although no special precautions were taken to prevent the formation of the granules that are considered to be fixation artifacts (Mancini, 1948). The deep maroon coloration obtained with Montagna's method was usually so dense that one could not tell whether the reaction was diffuse or granular. But in follicles with less glycogen the reaction was sometimes diffuse and sometimes granular, the granules often being disposed around the periphery of the cell. In follicles giving a slight to moderate reaction, the glycogen was often concentrated in the inner part of the outer sheath, adjacent to the inne sheath.

The stalk of the outer sheath connecting a 'capsule' to a follicle (Ryder, 1956) usually had large amounts of glycogen, even though the rest of the oute sheath might have little glycogen. In some preparations glycogen was found in the Huxley layer of the inner sheath. This agrees with the findings of Tamate (1951), who claimed to have detected glycogen combined with a protein in the Huxley layer of several mammals. There was little glycogen in

e bulb, that present being mainly in the cells of the proximal continuation
the outer sheath.

With Best's carmine and the Hotchkiss method, no glycogen was found in
e fibre except in the medulla. But when Montagna's modification was used
ycogen was often found in both cuticle and cortex. The bulk of the glycogen
the fibre was between the bulb neck and the pre-keratinization region,
hough traces were sometimes seen below this in the cells of the bulb, even
low as the first stratum above the papilla. Montagna, Chase, and Lobitz
952) said, on the contrary, that the glycogen appeared suddenly several
ls above the papilla. No reaction was obtained in the fully keratinized part
the fibre. There were greater variations in the amount of glycogen in the
re than in the outer sheath, and in some follicles the variations in the fibre
re associated with those in the sheath. The reaction of the fibre was streaky,
e glycogen apparently being in the outer parts of the cortical cells. No
action was observed in the intercellular spaces, which is interesting because
th the Hotchkiss method the first indication in the present study of poly-
ccharides in the non-keratinized part of the fibre was an intercellular
action. The glycogen reaction began at a lower level on one side of the fibre,
d at higher levels there was a tendency for it to be denser on one side. In
me follicles the reaction was only at one side over the whole of its length,
en starting immediately above the papilla. These observations provide
rther evidence that the bilateral nature of the fibre starts at a low level.

Slides left in 70% alcohol instead of periodic acid did not give any dense
aroon reaction considered to be due to glycogen. Instead the lower parts of
e fibre stained a dense magenta colour which was continued as streaks into
e fully keratinized part, and the inner sheath stained pink. Slides left in
astase for an hour before being put in the periodic acid did not show any
ycogen at all in the outer sheath, but in the fibre a few maroon streaks
casionally persisted; otherwise the fibre was of a similar colour to those
hich had been in 70% alcohol. Slides left for a longer time in the periodic
d often had fibres with a magenta stain in the fully keratinized part (com-
re Scott, 1953). But this was on only one side of the fibre, shown by staining
ernate sections with 'Sacpic' (Auber, 1952) to be the orthocortex.

With all methods used, traces of glycogen were found to persist in the
edulla of the fully keratinized part of the fibre. No detailed observations
re made of the glands, but in some preparations glycogen was seen in both
e sebaceous and sweat glands (compare Montagna, Chase, and Lobitz, 1952).

e of labelled glucose

The results obtained from the first experiment have already been sum-
arized (Ryder, 1957*a*). In the second experiment longer exposures were
owed and photographic grain counts were made, as described by Ryder
957*b*). Grains were counted in the autoradiographs of the bulb, of the lower
rt of the fibre, and of the region of positive reaction for glycogen in the
ter sheath. Unlike the results obtained by injecting cystine labelled with

sulphur-35 (Ryder, 1957*b*), there was little difference in the amount of radioactivity in these different regions of the follicle at each sampling time. For this reason the results of the individual counts are not given, but only a mean figure from all the regions (112 days' exposure) is shown below.

In the mouse killed 1 h after injection there was moderate activity in the bulb and the counts showed that there was a similar amount in the lower part of the fibre, but there was definitely less in the region of the outer sheath with a positive reaction for glycogen. The mean grain density was 56. Eight hours after injection the mean grain density had reached 95 and the glycogen seemed to contain most radioactivity and the bulb least.

The moderate amount of radioactivity found in the lower part of the fibre in the 8-h sample appeared in the fully keratinized part after 24 h. Otherwise the activity was declining in the lower part of the follicle in the 24-h and 3-day samples, the grain densities being 65 and 35 respectively. In the first experiment no activity could be detected in the follicles of the mouse killed 10 days after injection.

Tests for enzymes that are probably associated with glycogen

Acid phosphatase. With the lead-phosphate method a reaction was obtained with thick frozen sections but not with wax sections. The densest (black) reaction was in the lower part of the fibre up to about the level of the pre-keratinization region (fig. 1, C, D), but a brown reaction was obtained in the cytoplasm of the outer sheath cells. Most of the nuclei gave a positive reaction, as with alkaline phosphatase, and an apparent dense reaction in the upper part of the bulb might have been due to the close packing of the nuclei there. But there was neither a nuclear nor a cytoplasmic reaction in the lower part of the bulb. Often the papilla gave a positive reaction, and sometimes the capillaries too.

The orange-red reaction obtained with the azo-coupling technique was far superior. The papilla and nuclei were negative and the bulb gave little or no reaction, the densest reaction again being in the lower part of the fibre. This was between the neck of the bulb, at which level the reaction began gradually, and about the level of the pre-keratinization region where the reaction ended fairly abruptly, often asymmetrically. There was a reaction in the medulla which persisted beyond the level at which the cortex was

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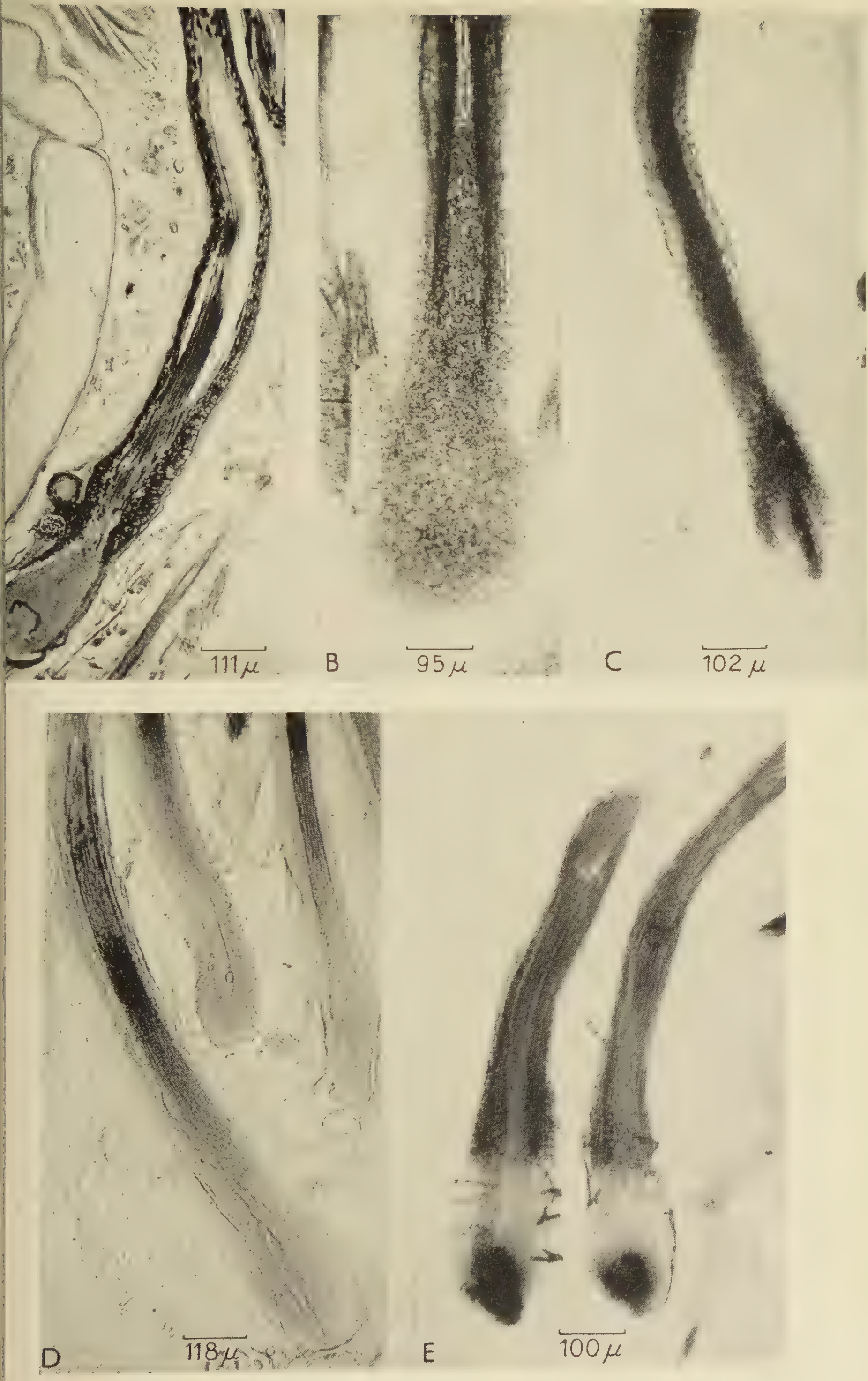
FIG. 1 (plate). A, wool follicle showing glycogen in the outer sheath and the lower part of the fibre; Montagna's modification of the Hotchkiss method. Note the 'capsule'; these are thought to be milia (benign tumours described in the human by Epstein and Kligman, 1956).

B, autoradiographic preparation of a vibrissal follicle from a mouse killed 8 h after an injection of glucose labelled with carbon-14. The photographic grains indicate radioactivity in the bulb, outer sheath, and lower part of the fibre. 50-μ frozen section, Kodak A.R. 50 film.

C and D, wool follicles in which the lead-phosphate method has been used to show acid phosphatase. C, recently fixed skin showing a dense reaction in the fibre. D, skin left in 5% formalin for about a year, showing a dense reaction only at about the pre-keratinization level with a gradually decreasing reaction below this.

E, two wool follicles in which the calcium-cobalt method has been used to show alkaline phosphatase. There is a reaction in the outer sheath, in the papilla (diffused into the bulb) and in capillaries surrounding the follicle.



M. L. RYDER

atinized. The inner sheath gave a very weak reaction which became derately dense at the distal limit of the sheath. The outer sheath, too, had a y pale reaction, but this became denser above the opening of the sebaceous nd, the reaction here being a continuation of a dense reaction in the epi- mis. Both glands gave a reaction, the reaction of the sweat gland being ater than that of the sebaceous gland, and often quite dense. The arrector scle gave little or no reaction.

n order to be sure that non-specific staining was not occurring, the fast e B salt was tried on control slides with no substrate. In fact a general pale low reaction was obtained in most parts of the follicle, but this could not confused with the orange-red reaction in the fibres. It might, however, ke interpretation of a weak reaction, such as that in the outer sheath, ficult. Red blood corpuscles were stained orange, possibly owing to coupling h histidine.

Alkaline phosphatase. Both wax and frozen sections gave a reaction with the cium-cobalt method, but that obtained with frozen sections was stronger, densest reaction being in the papilla. There was a weak reaction in the lb with some dense patches that were clearly due to diffusion from the illa. The outer sheath gave a reaction beginning at the neck of the bulb and rresponding with the strongest reaction for glycogen, but in only a few licles was this as dense as in fig. 1, E. The reaction was distributed through- the full width of the sheath; it was never found only in the outer part as icted in the mouse by Hardy (1952). There was occasionally a slight ction in the lower part of the fibre, and capillaries gave a strong reaction. e nuclei always gave a dense reaction even when the cytoplasm was only akly stained, but this is generally recognized (Pearse, 1953) as being a false ction. The inner sheath, too, gave a false reaction (present in control slides) mpare Hardy, 1952).

The azo-dye method for alkaline phosphatase produced a general yellowing the epidermis, follicle bulbs, and outer sheaths, which must be regarded non-specific staining because the same result was obtained when no sub- ate was added. This may be due to breakdown products of the azo com- und, or even to a coupling with certain amino-acid groups in the tissue. th α-naphthyl diazo naphthalene-1:5-disulphonate (Manheimer and Selig- n, 1948), capillaries, sweat glands, and the lower parts of the fibres gave a l-brown reaction. This was not obtained in control slides and was presum- ly due to alkaline phosphatase. That in the fibre terminated in a dense nd with a sharp distal limit at about the pre-keratinization level. The only ction in the papilla was that given by capillaries, but it was no denser here n elsewhere. With fast blue B and fast blue RR the colour was grey, but re was no reaction in the fibre.

DISCUSSION

The results of the present study confirm the observation of Montagna, ase, and Lobitz (1952) that there is glycogen in the unkeratinized part of the fibre. It was not always found, however, because of difficulties of demonstration or variations in the material. Care has to be taken in interpreting th Hotchkiss reaction when stronger oxidation is used (Lhotka, 1953), especiall as under such conditions even the fully keratinized part of the fibre gives magenta colour. But the reaction obtained in the present study in the unkeratinized part of the fibre is almost certainly due to glycogen, because lik the glycogen of the outer sheath it was deep maroon and was largely prevente when the sections were soaked in diastase solution before oxidation. Tha glycogen is only revealed in the fibre by stronger oxidation and is not entirely removed by diastase suggests that it is combined possibly as a glycoprotein, like that which Tamate (1951) claimed to have found in the inne sheath.

The presence of glycogen in the fibre before keratinization provides possible source of the carbohydrate in the fully-formed fibre (Bolliger an McDonald, 1949); this has usually been thought to be intercellular and t consist of substances trapped on keratinization.

Ryder (1957*a*) discussed the probable function of glycogen as a local stor of energy, that in the outer sheath to be passed to the papilla as glucose b the blood-vessels of the follicle, and that in the fibre to provide energy fo reactions associated with keratinization. The results of the experiments i which labelled glucose was injected threw some light on this problem. I has already been stated (Ryder 1957*a*) that the radioactivity in the bulb wa likely to be glucose or a compound such as glucose-1 or 6-phosphate deposite there directly (at any rate in the 1-h sample) to provide energy for mitosis The radioactivity in the fibre could, in addition to this, have been due t amino-acids (Black, Kleiber, and Baxter, 1955) or glycogen, although it i unlikely that much amino-acid could be synthesized within an hour and i none of the autoradiographic preparations was there a reaction for glycoge in the fibre. The radioactivity in the outer sheath was only associated with positive reaction for glycogen and was almost certainly due to this. The pea of radioactivity in the outer sheath after 8 h therefore suggests that the injecte glucose took several hours to be deposited there as glycogen. Althoug sufficient glycogen is supposed to be deposited in the early stages of th growth cycle of the mouse to provide for subsequent hair growth (Loewentha and Montagna, 1955), this shows that glycogen is still being deposited whil the hair is growing. After 8 h, however, the radioactivity declined at the sam speed as that in the rest of the follicle and there was no evidence that thi had been transferred to another part of the follicle. Some rough preparation made of the liver in the first experiment suggested that there the peak o activity occurred 24 h after injection.

With the lead-phosphate method for acid phosphatase one must beware o inaccurate localization and false reactions such as that seen at the cut ends o fibres. The azo-dye method, however, confirmed the reaction in the unkeratinized part of the fibre, and this agrees with the observations of previou authors on other animals. The reaction in the upper part of the bulb foun th the lead-phosphate method and by Moretti and Mescon (1956) was not nfirmed.

Braun-Falco (1957) discussed various possible functions of the acid osphatase in the fibre, but it is thought in the present study that an acid osphatase could be associated with the breakdown of the glycogen demon-ated in the same region of the fibre. Similarly with the alkaline phosphatase e would expect the greatest concentration of the enzyme to be associated th glycogen, so that the presence of an alkaline phosphatase in the outer eath would be reasonable. This could not be confirmed with the azo-dye ethod; so the reaction with the calcium-cobalt method may be due to fusion from the surrounding vessels. The suggestion of Durward and Rudall 949) that the papilla reaction could merely be a reaction of the capillaries borne out by the present work. Hardy (1952) discussed the possibility that e function of alkaline phosphatase was in the transport of substances. The nction of phosphatases in the glycogen cycle is to break down glucose-1-osphate and glucose-6-phosphate and it is possible that glucose can be in s form in the blood (at any rate for the short distance from the outer sheath) d that alkaline phosphatase releases glucose from these compounds in the pilla. Braun-Falco (1957) has in fact found a specific glucose-6-phosphatase the papilla of the human hair follicle and the results of the injection periments in the present study suggest the presence of glucose in the bulb.

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REFERENCES

BER, L., 1952. Trans. Roy. Soc. Edin., **62**, 191.

ACK, A. L., KLEIBER, M., and BAXTER, C. F., 1955. Biochem. Biophys. Acta, **17**, 346.

LLIGER, A., and MCDONALD, N. P., 1949. Aust. J. exp. Biol. med. Sci., **27**, 223.

AUN-FALCO, O., 1956. Arch. Klin. exp. Derm., **202**, 163.

— 1957. *The histochemistry of the hair follicle.* Proc. British Soc. for Research on Ageing: Symposium on the Biology of Hair Growth. (In the press.)

ASE, H. B., RAUCH, H., and SMITH, V. W., 1951. Physiol. Zool., **24**, 1.

NIACH, L., and PELC, S. R., 1950. Brit. J. Radiol., **23**, 184.

RWARD, A., and RUDALL, K. M., 1949. J. Anat. Lond., **83**, 325.

IS, W. J., GILLESPIE, J. M., and LINDLEY, H., 1950. Nature, **165**, 545.

STEIN, W., and KLIGMAN, A. M., 1956. J. Invest. Dermat., **26**, 1.

RDY, M. H., 1952. Amer. J. Anat., **90**, 285.

TCHKISS, R. D., 1948. Arch. Biochem., **16**, 131.

INSON, P. L., BUTCHER, E. O., and BEVELANDER, G., 1945. Anat. Rec., **93**, 355.

OTKA, J., 1953. Nature, **171**, 1123.

EWENTHAL, L. A., and MONTAGNA, W., 1955. J. Invest. Dermat., **24**, 429.

NCINI, R. E., 1948. Anat. Rec., **101**, 149.

MANHEIMER, L. H., and SELIGMAN, A. M., 1948. J. Nat. Cancer. Inst., **9**, 181.

MONTAGNA, W., CHASE, H. B., and HAMILTON, J. B., 1951. J. Invest. Dermat., **17**, 147.

—— —— and LOBITZ, W. C., Jr., 1952. Anat. Rec., **114**, 231.

MORETTI, G., and MESCON, H., 1956. J. Invest. Dermat., **26**, 347.

PEARSE, A. G. E., 1953. *Histochemistry, theoretical and applied*, London (Churchill).

RYDER, M. L., 1956. J. Agric. Sci., **47**, 187.

—— 1957*a*. *Nutritional factors influencing hair growth.* Proc. British Soc. for Research on Ageing: Symposium on the Biology of Hair Growth. (In the press.)

—— 1957*b*. Submitted for publication.

SCOTT, H. R., 1953. Nature, **172**, 674.

TAKEUCHI, T., and KURIAKI, H., 1955. J. Histochem. Cytochem., **3**, 153.

TAMATE, H., 1951. Tokohu J. Agric. Res., **2**, 31.

A Note on the Preservation of Adipose Lipid

By J. T. Y. CHOU

(*From the Cytological Laboratory, Department of Zoology, University Museum, Oxford*)

SUMMARY

The lipid globules of adipose cells become insoluble in fat solvents after they have een fixed in a suitable fixative and postchromed for 7 weeks at 37° C. The potassium ichromate acts merely as an oxidizing agent: little or no chromium is left in the lobules.

IN a previous paper (Chou, 1957) it was shown that adipose lipid is rendered insoluble in a fat solvent (xylene) by postchroming for 7 weeks at 37° C. his fact can be demonstrated by an experiment carried out on paraffin ections with Sudan colouring reagents. The present investigation was underaken to find out whether chromium combined with the adipose lipid, or hether the potassium dichromate merely rendered the lipid insoluble in at solvents by oxidation.

Cain (1947) reinvestigated Baker's acid haematein method (1946) and ound that the reaction is only given in the presence of chromium. He used iphenyl-carbazide to detect chromium.

When the staining technique of Baker's acid haematein method was applied o sections of the skin of the mouse that had been fixed in Ciaccio's fluid for days and then postchromed for 7 weeks, the result was, in general, negative. n certain parts of the sections, however, especially in those adipose cells ituated at the edge of the sections, the reaction was very weakly positive; the olour was sky-blue.

Other similar sections were tested for the presence of chromium by the se of diphenyl-carbazide. A saturated solution of this substance in 90% cohol was used. With this reagent, a red to violet colour indicates the pre- nce of chromium. Each section was washed in distilled water, laid on a slide, overed with a few drops of diphenyl-carbazide solution and examined im- ediately under the microscope. The result was negative both in the postromed tissue and in the control sections that had not been postchromed, ough certain adipose droplets near the edge of the section of the postromed tissue were very slightly pinkish or light orange.

From the above experiments the conclusion can be drawn that chromium as not present in most of the adipose globules of the skin of the mouse hich has been postchromed for 7 weeks, though a few globules near the rface of the piece of tissue contain a very small quantity. Experiments with dan colouring agents show that there is no more lipid in the globules that ntain a little chromium than in those that do not. It is evident, therefore, at the presence of chromium is not necessary for fixation of adipose lipid.

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It is to be presumed that potassium dichromate fixes the adipose lipid b
oxidation.

A piece of the small intestine of the mouse was fixed in formaldehyd
calcium and postchromed for 7 weeks, and the sections were treated by th
staining technique of Baker's acid haematein test. Examination showed th
the mitochondria were strongly coloured; so also were certain parts of th
cytoplasm. This result shows that a long period of postchroming does n
destroy the phospholipids of the mitochondria; however, the mitochondr
cannot be demonstrated in paraffin sections by Metzner's method after th
prolonged postchroming.

REFERENCES

Baker, J. R., 1946. Quart. J. micr. Sci., **87**, 441.
Cain, A. J., 1947. Ibid., **88**, 467.
Chou, J. T. Y., 1957. Ibid., **98**, 431.

The Histochemistry of Bound Lipids

By M. C. BERENBAUM

(*From the Glaxo Laboratories Ltd., Greenford*)

With one plate (fig. 1)

SUMMARY

Many tissue-lipids are bound in varying degrees to other substances, mainly protein, and so are resistant to extraction by organic solvents. Such bound lipids are still present in paraffin sections, but they cannot be satisfactorily demonstrated by conventional use of the Sudans. It has been found that an essential condition for such demonstration is their prior dissociation from their complexes by hydration, heat, or the action of proteolytic enzymes.

Methods are described by which, after routine fixation, bound lipids may be partially dissociated from their complexes and then demonstrated by Sudan black, in paraffin sections or in smears.

These methods show lipids to be present constantly in association with nuclear and cytoplasmic nucleoprotein, in basement membranes, epithelial brush borders, neurones, and many other structures. There is evidence that these lipids do not form a homogeneous class, but vary widely in their physico-chemical natures.

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THE HISTOCHEMICAL BASIS OF THE METHODS

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INTRODUCTION

TISSUE-lipids may be classified not only by chemical constitution, but also by the degree to which they are bound to other substances. Although in the latter property a continuous range exists, it is helpful to consider three main groups of lipid.

(*a*) The lipids with which the histologist is most familiar are those with little or no binding. These are readily demonstrable by conventional methods, e.g. with Sudan IV in 70% alcohol, and rapidly extracted by fat solvents. They include the fat of adipose tissue and of the adrenal cortex, the secretion of sebaceous glands, and the droplets produced in fatty degeneration.

(*b*) Lipids showing between weak and moderately strong binding to other

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substances are present in myelin sheaths, erythrocytes, the granules of eosino phil and neutrophil leucocytes, mycobacteria, the isotropic disks of skeleta muscle, lipofuscins, and the cytoplasm of many cells. Some of these ar weakly coloured by red Sudans; all are well shown by Sudan black B in 70° alcohol, especially at elevated temperatures. The partial binding of thes lipids enables them to be fixed sufficiently to resist routine processing an embedding in paraffin wax. Simple formaldehyde fixation may be adequat (Pearse, 1953), but better results are obtained if this is used with calcium, cadmium, or cobalt salts, or dichromate (Ciaccio, 1934; Baker, 1946 McManus, 1946). These lipids may often be stained by chemical method depending on mordanting or oxidation and subsequent staining with haematoxylin (Baker, 1946; Ciaccio, 1954), Schiff's reagent (Dixon and Herbertson 1950 *a*, *b*; Pearse, 1953), or ammoniacal silver (Dixon, 1953), and some o them are responsible for the acid-fast staining of certain structures (Berg 1953 *a*, *b*, *c*, 1954).

(*c*) Finally, firmly bound lipids are found in cell nuclei, reticulin, epithelia brush-borders, &c. Although little evidence exists so far as to their function their distributions suggest important roles in tissue metabolism and structure Because of their close spatial relationship or chemical combination with th substances to which they are bound, it is difficult or impossible to demonstrat them by conventional histological methods (Lison, 1953). Though much lipi may be extracted from tissues by organic solvents, for this it is necessar that the tissues be finely divided, and, further, for the removal of maxima amounts, acid or alkali must be added to the solvent. It is evident that, whe pieces of tissue of the size used for histological processing are first fixed an then passed through a few changes of neutral organic solvents and brough into paraffin wax, significant quantities of bound lipid must be retained i the tissue. It also follows that absence of simple sudanophilia in frozen section is no proof of the absence of lipids, as seems to be assumed by many authors

The purpose of this paper is to present three methods, two of which hav been briefly reported previously (Berenbaum, 1954), by which bound lipid may be easily demonstrated in paraffin sections or in smears.

The known insolubility of phosphatides in acetone was the basis of th experiments that led to the evolution of method A. Method B was discovere in attempting to apply Sheehan and Whitwell's (1949) method for tubercl bacilli to paraffin sections.

METHODS

Method A

Acetone Sudan black

(1) Fix in formaldehyde solution, Zenker, ethanol, Carnoy, &c.

(2) Mount paraffin sections on albuminized slides, and take through xylen and alcohol to water. Remove mercuric precipitate if necessar Smears may be fixed in formalin vapour, methanol, Zenker, &c., an transferred straight to water.

(3) Leave in running tap-water for 2–48 h. Overnight washing usually gives satisfactory results.

(4) Rinse in acetone.

(5) Place in a saturated solution (about 2% w/v) of Sudan black B or acetylated Sudan black (Lillie and Burtner, 1953; Casselman, 1954) in acetone at 37–60° C.

(6) Coloration is progressive. Four hours is usually adequate, but longer or shorter times (1–48 h) are sometimes advantageous.

(7) Wash in xylene till no more colouring agent is extracted (10 min. or more).

(8) Mount in balsam or D.P.X.

Method B

Burnt Sudan black

(1) to (3) as above. Alternatively to washing the sections, they may be kept at 60°–70° C overnight before de-waxing.

(4) Rinse in 70% ethanol.

(5) Place horizontally on a staining rack and cover with a freshly filtered, saturated solution of Sudan black B, or of its acetylated derivative, in 70% ethanol. Set the solution alight and allow it to burn out. Drain off the residue and, without allowing the sections to dry, rapidly cover with fresh solution and relight. Repeat this 6–12 times (6 times is usually adequate.)

(6) Wash in absolute ethanol until no more colouring agent is extracted (10 min or more).

(7) Clear in xylene. Mount in balsam or D.P.X.

Method C

(1) to (3) as above.

(4) Rinse in 70% ethanol.

(5) Place in a saturated solution of Sudan black B in 70% ethanol at 60° C.

(6) Coloration is progressive. Four hours is usually adequate.

(7) Rinse in 70% ethanol.

(8) Transfer to absolute ethanol for about 10 min.

(9) Clear in xylene. Mount in balsam or D.P.X.

Notes

(1) The Sudan black solution in method A is kept in an airtight jar, sealed with silicone grease, in the incubator. Slow losses from evaporation are made up by periodically 'topping up' with acetone. It lasts for a year to 18 months at 37° C without evident deterioration, but after this time it produces progressively weaker colouring.

Immediately after preparation the solution tends to leave a deposit on slides, but this tendency is lost after a week or so.

(2) Sections coloured inadequately may be rinsed in acetone or 70% ethanol for methods A, B, and C, respectively, and the procedure is repeated from stage (5) onwards.

(3) The fixative and the times of stages (3) and (5) may be varied widely to suit the problem under investigation, the final result being much influenced by these factors.

(4) Poor results are often given by rapidly growing tumours or severely infected tissues. This is possibly due to ante-mortem or rapid post-mortem autolysis, when the free lipid content of the tissues rises (Sperry, Brand, and Copenhaver, 1942) and the bound-lipid content presumably decreases.

(5) Intensified colouring results if sections of material fixed in formaldehyde solution are treated with 0·01% trypsin in buffer at pH 7 and 37° C for 1 h before washing.

Results

The colours obtained are usually black or grey, but a polychromatic effect is often observed, some structures becoming blue or brown, especially when acetylated Sudan black is used.

Structures shown by methods A and B

Chromatin, mitotic chromosomes, and nuclear membranes are well shown, especially by burnt Sudan black (fig. 1, A). Cytoplasm is coloured with an intensity proportional to its content of ribonucleic acid. The inclusion bodies of ectromelia and myrmecia and the leucocytic inclusions seen in disseminated lupus erythematosus are shown by these methods; these objects contain nucleic acid.

Both methods colour the cytoplasmic granules seen in macrophages, in the granular-cell myoblastoma, and in the renal tubular epithelium of the rat and new-born human being.

In skeletal muscle the isotropic and M-bands are well shown (fig. 1, B).

Erythrocytes and the cytoplasm of developing red cells are deeply coloured by burnt Sudan black, but only faintly by acetone / Sudan black. As with other lipid methods, the periphery of the granules of eosinophil leucocytes is well shown.

FIG. 1 (plate). A, salivary gland chromosomes of larva of *Drosophila*, fixed in formaldehyde solution, washed 18 h, paraffin section, burnt Sudan black.

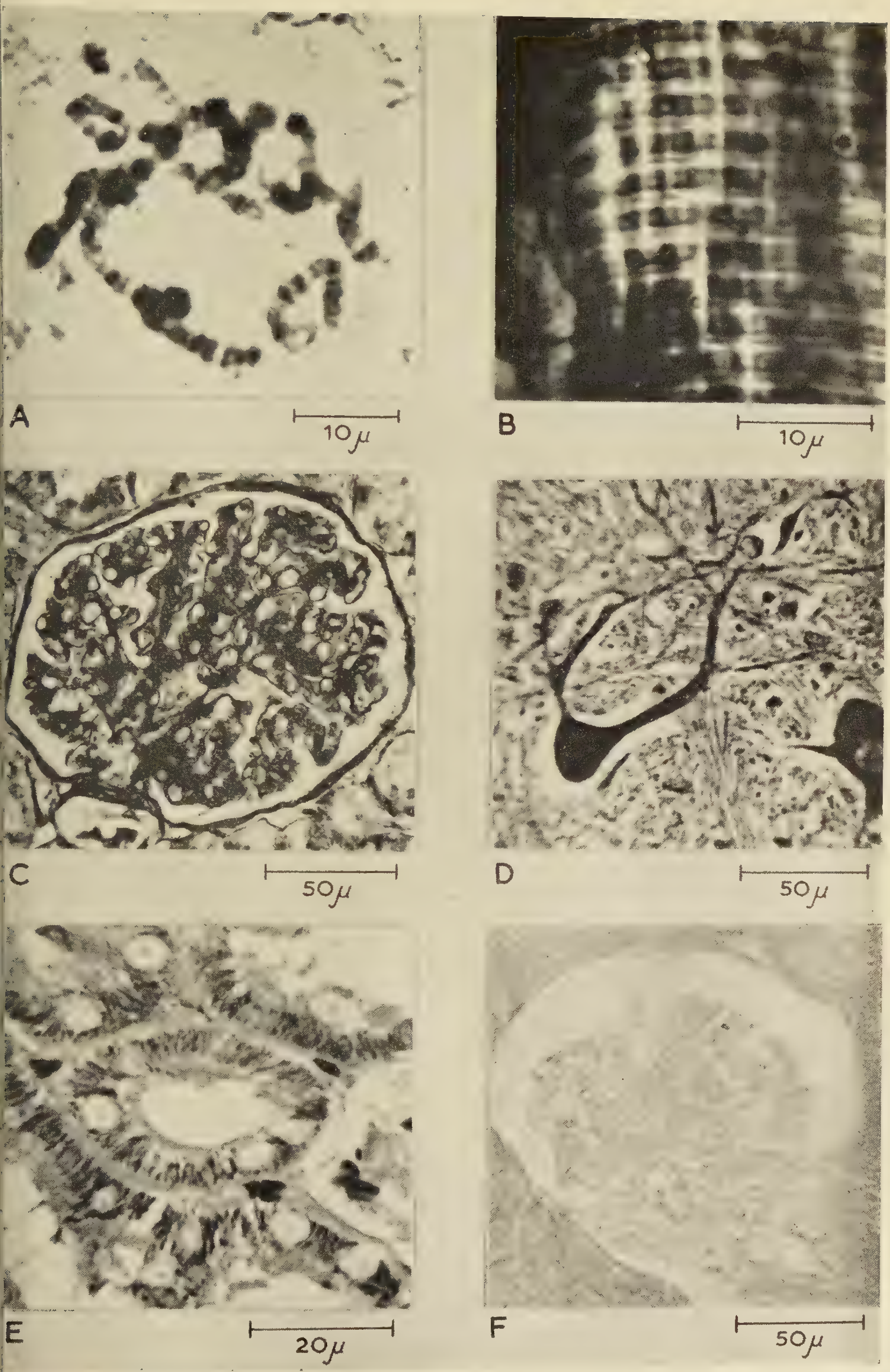
B, skeletal muscle, showing deep coloration of the isotropic and M-bands; washed 18 h, acetone / Sudan black 4 h.

C, kidney, showing intense coloration of basement membranes of glomerulus, tubules, and Bowman's capsule. Faintly coloured red blood-corpuscles are seen in some glomerular capillaries. Washed 18 h, acetone / Sudan black 4 h.

D, medulla oblongata, showing deeply coloured nerve-cell bodies and processes; washed 18 h, acetone / Sudan black 4 h.

E, rat kidney, showing coloration of mitochondria in the convoluted tubules. Red blood-corpuscles in the intertubular capillaries and in the glomerulus at the right-hand side of the illustration are also deeply coloured. The nuclei are only faintly coloured. Trypsin 0·1 mg/ml pH 7, 37° C, 1 h; washed 18 h; Sudan black in 70% ethanol at 60° C, 4 h.

F, kidney, unfixed tissue extracted for 70 days in chloroform-methanol before embedding; washed 18 h, acetone / Sudan black 7 h. Almost all lipid has been extracted. Compare with C.



M. C. BERENBAUM

Other structures giving a positive reaction include thyroid colloid, some ucins, cartilage ground-substance, protein casts in renal tubules, bacteria, nd lipofuscins.

dditional structures shown by acetone / Sudan black

This method gives intense coloration of basement membranes and of owman's capsule in the kidney (fig. 1, c), and moderate or deep coloration of ollagen, reticulin, and elastic fibres.

Nerve-cells and their processes are well shown in the brain stem, spinal ord, and peripheral nerve (fig. 1, d). Delineation is not so sharp in the more ephalic parts of the nervous system or in the cerebellum. Neuroglial cells and heir processes may be moderately coloured, especially in pathological material.

Epithelial brush borders are often deeply coloured.

Other structures shown include amyloid deposits, fibrin, 'fibrinoid' of heumatic nodules, blood plasma, megakaryocytic cytoplasm, blood platelets, he cytoplasm and granules of neutrophil leucocytes, corpora amylacea, ituitary colloid, the 'morula' bodies of cerebral trypanosomiasis, and smooth uscle cytoplasm.

Tuberculous giant cells may show a characteristic zoned structure, with eripheral and central dark areas and an intermediate pale area that sometimes hows faint radial striation.

tructures shown by method C

Nuclear chromatin is faintly shown.

In certain tissues mitochondria are well demonstrated, e.g. in renal con-oluted tubules and cardiac muscle (fig. 1, e). Erythrocytes and the cytoplasm f developing erythroid cells are deeply coloured. In the nervous system yelin sheaths are well shown, and axon sheaths and neuronal bodies are aintly coloured. Neuronal end-feet are demonstrated around anterior horn ells in the spinal cord. Other structures shown include the cytoplasm of mooth muscle and megakaryocytes, striations of skeletal muscle, bacteria, he striated border of intestinal epithelium and zymogen granules in the astric glands.

THE HISTOCHEMICAL BASIS OF THE METHODS

xtraction techniques

Initially the extraction techniques of Baker (1946) and Pearse (1953) were sed, but, except with a few structures, e.g. red cells, the results were hardly ffected. It was found that in order to diminish markedly the bound-lipid ontent of tissues, as shown by these methods, it was necessary to carry out oxhlet extraction of thin slices (1 mm thick or less) of unfixed tissue for at east 2 weeks in methanol-chloroform mixture (1/3). After 70 days' extraction n this mixture or in a mixture of equal volumes of methanol, chloroform, and ichlorethylene, there was practically no demonstrable lipid left in most ssues (fig. 1, f). The structure of such exhaustively extracted tissues was remarkably well preserved, as shown by staining with haematoxylin and eosi or other routine stains.

Certain structures were rather resistant to extraction, notably muci corpora amylacea, collagen, and reticulin. In fact 70 days' extraction did no remove the lipids demonstrable with acetone / Sudan black from muci This may have been due to carbohydrate bonds, which are very resistant t splitting by organic solvents (Uyei and Anderson, 1931; Anderson, Reeves and Stodola, 1937; Lovern, 1952).

Action of proteolytic enzymes

Proteolytic enzymes have for many years been used as 'unmasking' agent for lipids (Parat, 1928).

Accordingly, Carnoy-fixed sections of lymph-node were treated with 0·2% solution of pepsin in 0·02 N hydrochloric acid at 37° C for 30 min They showed good coloration with burnt Sudan black without furthe washing, whereas control sections treated with water or 0·02 N hydrochlori acid were only faintly coloured.

Treatment of sections of alcohol-fixed intestine with 0·01% trypsin at pH and 37° C for 1 h, with subsequent overnight washing, removed all materia demonstrable by acetone / Sudan black or burnt Sudan black.

However, even prolonged treatment (e.g. 19 h) with pepsin did not preven colouring with acetone / Sudan black or burnt Sudan black.

Pepsin leaves in the nucleus a protein framework (Mazia, 1941, 1950) t which some lipid is presumably bound, but removal of protein with trypsi appears to result in loss of all attached lipid, just as it results in the loss o attached nucleic acid (Kaufmann, Gay, and McDonald, 1950).

Formaldehyde fixation renders tissue proteins somewhat more resistant t trypsin than does fixation in alcohol, and it was found that coloration o formaldehyde-fixed sections with any of the three methods was usually mor intense if washing had been preceded by treatment with trypsin for 1 h.

Action of soaps

Following the method of Tayeau (1939 *a*), who showed that lipoprotei were dissociated by soaps, I treated sections of ethanol-fixed spinal cord an intestine and formaldehyde-fixed brain with 1% sodium ricinoleate in buffe at pH 7 and 37° C for 1 h. They were well coloured by acetone / Sudan blac without further washing, whereas control sections in buffer were coloure very weakly.

Relation to nucleic acids

Since all structures known to contain nucleic acids were also well shown b methods *A* and *B*, it was necessary to establish that the substances responsibl were not nucleic acids themselves but the associated lipids.

Sections of Carnoy-fixed lymph-node were incubated at 37° C in 0·001% desoxyribonuclease at pH 7 for 1 h, with 0·003 molar magnesium sulphat (McCarty, 1946), after which they were Feulgen-negative. After overnigh ashing the nuclear chromatin showed slightly reduced colouring with etone / Sudan black and burnt Sudan black. The nucleoli were unaffected.

Similarly, sections of alcohol-fixed spinal cord were incubated with 0·1% bonuclease in water at 37° C for 1 h. There was a similar, slight reduction coloration of nucleoli and Nissl bodies.

Conversely, in sections of tissues extracted in organic solvents until the uclei were no longer coloured by acetone / Sudan black or burnt Sudan ack, Feulgen staining was as intense as in unextracted tissues. It may be ncluded from these experiments that coloration by these two methods of ructures containing nucleic acid is due to associated lipid. Since treatment ith nucleases reduces but does not abolish the positive reaction, it may be esumed that some lipid is bound directly to nucleic acid and is therefore moved with it. The rest is probably bound to protein, as is suggested by the tion of proteolytic enzymes.

vitro *tests*

Substances known to contain lipoproteins, e.g. brain thrombo-plastin or sample of hepatic liponucleoprotein (Greenstein and Jenrette, 1940), were loured by these methods when dried on slides and fixed in formalin vapour. olouring of phosphatides and acetone-soluble lipids of brain and red blood-rpuscles could not be demonstrated since they were dissolved off the slide some stage in the procedures, despite prolonged immersion in formalin. he difficulties of satisfactory *in vitro* testing are referred to further in the iscussion.

elation to paraffin inclusions

Nedzel (1953) showed that paraffin sections, if dehydrated in acetone after aining, might still contain paraffin in the form of birefringent nuclear clusions. These were not removed by xylene and could be coloured with l red O. The possibility therefore existed that such residual paraffin could responsible for nuclear coloration obtained with ASB or BSB, although it uld not explain the results with tissue smears.

However, when sections containing such inclusions were washed in water d subjected to the acetone / Sudan black and burnt Sudan black procedures, relationship was found between the distribution of inclusions and the final pearance of the treated section, nor did this appearance differ from that of milar sections without inclusions.

It may be concluded that such paraffin inclusions are not responsible for e colouring by these methods.

Discussion

Since many structures reacting positively to the methods here presented e not shown by other lipid-colouring methods in common use, it is relevant mention some of the chemical or physical evidence for the presence of lipid in these structures. This subject has been reviewed by Lovern (1952) Bloor (1943), and Wittcoff (1951).

Nuclear lipids have been obtained by extracting isolated nuclei in organic solvents (Stoneburg, 1939; Dounce, 1943; Williams and others, 1945; Wang and others, 1952; Carver and Thomas, 1952; Spiro and McKibbin, 1956). A lipoprotein was obtained from rat-liver nuclei by Wang, Mayer, and Thomas (1953). Lipid was extracted from mammalian sperm, consisting largely o nuclei, by Miescher (1897) and Dallam and Thomas (1952), and Berg (1954 showed that sperm heads contained an acid-fast lipid.

Although the nucleus differs little from the cytoplasm in its lipid conten (Williams and others, 1945), it is strange that most of the lipid in the forme is most effectively masked when other techniques are used, although smal amounts of nuclear lipid were detected histologically by Fels (1926), Mensinkai (1939), Serra (1947), Callan and Tomlin (1950), and Brock, Stowell, and Couch (1952), generally as sudanophil inclusions or in association with the nucleolus.

An analogous situation has been shown to exist in the adrenal gland where the medulla contains as much cholesterol as the cortex, although i can be demonstrated histologically only in the latter (Leulier and Revol 1930).

Berg (1951) denied that nuclei contained significant amounts of lipid. In formaldehyde-fixed frozen sections his benzpyrene method showed much cytoplasmic lipid but no appreciable quantity in the nuclei. Further, on examining preparations of isolated nuclei he found that around the benzpyrene-negative nuclei were fragments of lipid-laden cytoplasm or nuclea membrane. He therefore maintained that the lipid found on analysing such preparations was due to cytoplasmic contamination. However, Berg did no envisage that nuclear lipids might be so 'masked' as to defy demonstration by his technique. Chayen, La Cour, and Gahan (1957) have found that nuclea lipids can be demonstrated by Berg's method provided that a phosphatide precipitant, such as ammonium reineckate, is added to the formaldehyde solution in which the tissue is fixed.

Little and Windrum (1954) and Windrum, Kent, and Eastoe (1955), using extraction in organic solvents and X-ray diffraction, demonstrated a lipid in isolated reticulin.

Polarization microscopy has been used to show the presence of oriente lipids in the ground cytoplasm of various epithelial cells, and especially in th brush borders of intestinal, renal, and salivary duct epithelium (Hillarp and Olivecrona, 1946; Olivecrona and Hillarp, 1949). Molnar (1951) used simila methods to demonstrate lipids in corpora amylacea. Lipids are also shown in epithelial brush borders by Berg's (1951) benzpyrene method. McColl and Rossiter (1950) extracted a lipid from nerve axoplasm apart from that in th myelin sheath. Koechlin (1955) showed that axoplasm contained 25% by weight of a lipoprotein, whose lipid was unusually strongly bound to protein in that dissociation of the complex required treatment with hydrochloric o ichloracetic acid. Dixon and Herbertson (1950 *a*) and Dixon (1953) used xidation with periodic acid and subsequent treatment with Schiff's reagent or mmoniacal silver to stain cytoplasmic lipids in nerve-cells and their axons. t is interesting, in comparing their results with those obtained by the methods escribed here, that the periodic acid / silver method also stained Nissl bodies, lood-vessel reticulin, corpora amylacea, and Bowman's capsule. Wolman 955 *a* and *b*) has shown that lipids are responsible for the staining of euronal and neuroglial processes by silver impregnation methods.

Bound lipids have also been demonstrated histologically in bacteria by urdon (1946) and Sheehan and Whitwell (1949), in the isotropic disk of keletal muscle by Dempsey, Wislocki, and Singer (1946) and Berg (1951) and n blood- and marrow-cells by Hayhoe (1953).

Although most of the structures demonstrated by the methods outlined in his paper have already been shown to contain lipid by other means, the pecificity of these methods cannot yet be regarded as established beyond oubt. Prevention of colouring by previous extraction in fat-solvents does not nally prove the lipid nature of material responsible for colouring, since some rotein and other materials are removed by such treatment (Folch and van lyke, 1939; Christensen, 1939; Folch and Lees, 1950).

Proof of specificity for lipids can be supplied by comprehensive *in vitro* ests, such as were carried out by Casselman (1952) for Baker's acid-haematein tain. The difficulty here is that many preparations of biological substances nevitably contain varying amounts of bound lipids from which they may ometimes be freed only with difficulty. For instance, Kendall (1941) found hat 4-times-crystallized serum albumin contained 2–3% of lipid and 8-times-rystallized over 1%.

I have found that commercially obtained desoxyribonucleic acid, deposited n slides, gives a positive reaction with acetone / Sudan black. However, a ample boiled for 24 h in chloroform-methanol mixture yielded 1–2% of its eight as fatty material that could easily be coloured by Sudan IV in 70% lcohol. It is apparent that conclusive *in vitro* testing must depend on the reparation of a range of demonstrably lipid-free proteins and other materials, s well as a number of complexes of lipids with other substances.

The rationale of the methods is possibly as suggested below. It has been hown that many cellular lipoproteins exist in laminated form, with layers of ipid 'sandwiched' in between layers of protein (Schmitt and Bear, 1939; Chargaff, 1949; Frey-Wyssling, 1949; Sjöstrand, 1953), as, for instance, the ouble membranes of the endoplasmic reticulum and of mitochondrial cristae een under the electron microscope. Such lamination, or indeed any close hysical association of lipid with non-lipid substances, might effectively revent their taking up fat-soluble colouring agents by simple steric hindrance. artial dissociation of these complexes would allow access to such colouring gents. Such dissociation may be caused by simple contact with water (Jukes nd Day, 1932; Tayeau, 1939 *b*) and by the action of soaps (Tayeau, 1939 *a*), f proteolytic enzymes (Parat, 1928) or of dilute acid (Ackerman, 1952). In the methods described here, washing in water has been adopted as the simples and most controllable method of dissociation.

The mechanism of coloration in the burnt Sudan black method calls fo some comment. A clear physico-chemical picture of the process cannot be drawn at present, but it may be supposed that during the burning-off of the alcohol in the solution the Sudan black becomes super-saturated and, as it were, 'driven into' the lipid.

The claim put forward for these methods, that they demonstrate lipids is based on several pieces of evidence. With some isolated exceptions, all the structures demonstrated are known to contain lipid. Colouring can be prevented by exhaustive extraction with lipid solvents. Sections can be decolorized by leaving them for several days in organic solvents. The colouring agent is widely used in techniques specific for lipids. The finding that acetylated Sudan black may be used in these methods with at least as good results as the unacetylated substance is to some extent evidence that coloration is due to physical solution of the material in lipid and not to a chemical reaction between them (Lillie and Burtner, 1952, 1953; Casselman, 1954). Additional evidence is that the red Sudans may also be used, although colouring is much weaker. Finally, in order to colour sections with these methods it is necessary to subject them first to procedures known to split lipoproteins.

It must be admitted that none of these considerations lead to anything more than a strong presumption that what is being demonstrated is lipid; some of the objections to such a presumption have already been considered, but taken together they constitute fairly good evidence pointing to this conclusion.

It would appear that bound lipids should be thought of as existing in a wide variety of complexes differing physically and chemically. In the first place they differ in extractability, this probably reflecting differences in the firmness of binding. Secondly, each of the three methods produces a different picture, and other methods for demonstrating bound lipids (Baker, 1946; Berg, 1951; Berg, 1953 *a–c*, 1954, &c.) also differ in the degree to which they colour different structures. It follows that, just as it is now customary to use a variety of histochemical methods to characterize the more easily demonstrable lipids (e.g. sudanophilia, reaction with Nile blue, the acid haematein test, and the Liebermann–Burchardt reaction), so it is necessary to use a battery of methods in order to characterize the bound lipids found in any structure. Closer examination of the effect of varying treatments of the section, especially the use of enzymes, may be expected to further the histochemical analysis of bound lipids.

It is perhaps premature to attempt an explanation for the presence of lipid in many structures where it has been demonstrated. Lipids, by virtue of their hydrophobe qualities, play an important part in the make-up of natural semipermeable membranes. In this connexion the constant association of lipid with nucleoprotein is of great interest. It is evident that the complex activities in which nucleoproteins play a vital role, such as protein synthesis or mitosis, uire for their performance the orderly interaction of many cell components. may be suggested that, for such interaction to remain orderly, many cient but labile barriers must be constantly in operation, in order to prevent erference by inappropriate cell constituents. Such barriers might be ovided by lipid or lipoprotein membranes.

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REFERENCES

KERMAN, G. A., 1952. J. Nat. Cancer Inst., **13**, 219.
DERSON, R. J., REEVES, R. E., and STODOLA, F. H., 1937. J. biol. Chem., **121**, 649.
KER, J. R., 1946. Quart. J. micr. Sci., **87**, 441.
RENBAUM, M. C., 1954. Nature, **174**, 190.
G, J. W., 1953*a*. J. Histochem., **1**, 436.
— 1953*b*. Amer. J. Clin. Path., **23**, 513.
— 1953*c*. Yale J. Biol. Med., **26**, 215.
— 1954. Arch. Path., **57**, 115.
G, N. O., 1951. Acta path. scand., Suppl. **90**.
OR, W. R., 1943. *Biochemistry of the fatty acids and their compounds, the lipids.* New York (Reinhold).
CHET, J., 1950. Annals N.Y. Acad. Sci., **52**, 861.
CK, B., STOWELL, R. E., and COUCH, K., 1952. Lab. Invest., **1**, 439.
RDON, K. L., 1946. J. Bact., **52**, 665.
LAN, H. G., and TOMLIN, S. G., 1950. Proc. Roy. Soc. B, **137**, 367.
RVER, M. J., and THOMAS, L. E., 1952. Arch. Biochem. Biophys., **40**, 342.
SSELMAN, W. G. B., 1952. Quart. J. micr. Sci., **93**, 381.
— 1954. Ibid., **95**, 321.
ARGAFF, E., 1949. Discussions Faraday Soc., no. **6**, 118.
AYEN, J., LA COUR, L. F., and GAHAN, P. B., 1957. Nature, **180**, 652.
RISTENSEN, H. N., 1939. J. biol. Chem., **129**, 531.
CCIO, C., 1934. Boll. Soc. Ital. Biol. sper., **9**, 137.
— 1954. Bull. Micr. appl., **4**, 45, 97.
LLAM, R. D., and THOMAS, L. E., 1952, Nature, **170**, 377.
MPSEY, E. W., WISLOCKI, G. B., and SINGER, M., 1946. Anat. Rec., **96**, 221.
KON, K. C., 1953. J. Path. Bact., **66**, 539.
— and HERBERTSON, B. M., 1950*a*. Ibid., **62**, 335.
— —— 1950*b*. J. Physiol., **111**, 244.
UNCE, A. L., 1943. J. biol. Chem., **147**, 685.
— Ibid., **151**, 221.
S, E., 1926. Zbl. Gynäk., **50**, 35.
LCH, J., and VAN SLYKE, D. D., 1939. J. biol. Chem., **129**, 539.
— and LEES, M., 1950. Fed. Proc., **9**, 171.
EY-WYSSLING, A., 1949. Discussions Faraday Soc., no. **6**, 130.
EENSTEIN, J. P., and JENRETTE, W. W., 1940. J. nat. Cancer Inst., **1**, 91.
YHOE, F. G. J., 1953. J. Path. Bact., **65**, 413.
LLARP, N. A., and OLIVECRONA, H., 1946. Acta Anat., **2**, 119.
ES, T. H., and DAY, H. D., 1932. J. Nutrition, **5**, 81.

KAUFMANN, B. P., GAY, H., and McDONALD, M. R., 1950. Cold Spring Harbour Symp. quan
Biol., **14**, 85.

KENDALL, F. K., 1941. J. biol. Chem., **138**, 97.

KOECHLIN, B. A., 1955. J. biochem. biophys. Cytol., **1**, 511.

LEULIER, A., and REVOL, L., 1930. Bull. Hist. appl., **7**, 241.

LILLIE, R. D., and BURTNER, H. J., 1952. J. nat. Cancer Inst., **13**, 220.

—— —— 1953. J. Histochem., **1**, 8.

LISON, L., 1953. *Histochimie et cytochimie animales.* Paris (Gauthier-Villars).

LITTLE, K., and WINDRUM, G. M., 1954. Nature, **174**, 789.

LOVERN, J. A., 1952. *The mode of occurrence of fatty acid derivatives in living tissues.* Londo
(H.M.S.O.).

MAZIA, D., 1941. Cold Spring Harbour Symp. quant. Biol., **9**, 40.

—— 1950. Annals N.Y. Acad. Sci. **50**, 954.

McCARTY, M., 1946. J. gen. Physiol., **29**, 123.

McCOLL, J. D., and ROSSITER, R. J., 1950. Nature, **166**, 185.

McMANUS, J. F. A., 1946. J. Path. Bact., **58**, 93.

MENSINKAI, S. W., 1939. Ann. Bot., **3**, 763.

MIESCHER, F., 1897. *Die histochemischen und physiologischen Arbeiten.* Leipzig (Vogel).

MOLNAR, J., 1951. Nature, **168**, 39.

NEDZEL, G. A., 1951. Quart. J. micr. Sci., **92**, 343.

OLIVECRONA, H., and HILLARP, N. A., 1949. Acta anat., **8**, 281.

PARAT, M., 1928. Arch d'Anat. micr., **24**, 73.

PEARSE, A. G. E., 1953. *Histochemistry, Theoretical and Applied.* London (Churchill).

SCHMITT, F. O., and BEAR, R. S., 1939. Biol. Rev., **14**, 27.

SERRA, J. A., 1947. Cold Spring Harbour Symp. quant. Biol., **12**, 192.

SHEEHAN, H. L., and WHITWELL, F., 1949. J. Path. Bact., **61**, 269.

SJÖSTRAND, F. S., 1953. Nature, **171**, 30, 31.

SPERRY, W. M., BRAND, F. C., and COPENHAVER, W. C., 1942. J. biol. Chem., **144**, 297.

SPIRO, M. J., and McKIBBIN, J. M., 1956. Ibid., **219**, 643.

STONEBURG, C. A., 1939. Ibid., **129**, 189.

TAYEAU, F., 1939*a*. Compt. rend. Soc. Biol. Paris., **130**, 1027.

—— 1939*b*. Ibid., **130**, 1029.

UYEI, N., and ANDERSON, R. J., 1931. J. biol. Chem., **94**, 653.

WANG, T. Y., CARVER, M. J., RAMSEY, R. H., FUNCKES, A. J., and THOMAS, L. E., 195
Fed. Proc., **11**, 306.

—— MAYER, D. T., THOMAS, L. E., 1953. Exp. Cell Res. **4**, 102.

WILLIAMS, H. H., KAUCHER, M., RICHARDS, A. J., MOYER, E. Z., and SHARPLESS, G. R., 194
J. biol. Chem., **160**, 227.

WINDRUM, G. M., KENT, P. W., and EASTOE, J. E., 1955. Brit. J. exper. Path., **36**, 49.

WITTCOFF, H., 1951. *The Phosphatides.* New York (Reinhold).

WOLMAN, M., 1955*a*. Quart. J. micr. Sci., **96**, 337.

—— 1955*b*. Proceedings of the Second International Congress of Neuropathology, London
1, 65.

Synaptic Relations in the Atrial Nervous System of Amphioxus

By QUENTIN BONE

(*From the Department of Zoology, University Museum, Oxford*)

With three plates (figs. 3–5)

SUMMARY

A detailed description of the system of peripheral nerve-cells upon the gut and erticulum of amphioxus (*Branchiostoma*) is given; it is shown experimentally by ans of degeneration experiments that these cells are connected with the central vous system by their own axons, which run in the dorsal-root nerves. The form d connexion of the cells are described, special attention is paid to the problems of multinucleate cells in the plexus, and to the occurrence of possible asynaptic conxion between neighbouring nerve-cells. No sheath-cells have been observed upon peripheral nerve-fibres, either within the atrial plexus or upon the dorsal-root ve bundles; earlier misinterpretations of the nuclei of the cells of the epineurium und the dorsal nerve bundles are discussed. The origin of the atrial system in ogeny is discussed; it is suggested that it arises in an analogous manner to the eric plexuses of vertebrates, by outgrowth from the central nervous system. The t that this system of nerve-cells plays in the life of the animal is not known.

Finally, the relation of this system of cells to that found upon the guts of other ups of animals is discussed, and it is concluded that the system is not homologous h the enteric systems of nerve-cells in the vertebrates.

INTRODUCTION

T has long been known that there are peripheral nerve-cells in various positions around the atrium of amphioxus; they lie just underneath the er of ectodermal atrial epithelium that lines the atrium and covers the ferent organs in it. Such cells have been described by Dogiel (1902) and tchin (1913) upon the gill bars; by Holmes (1953) upon the atrio-coelomic nnels and within special papillae around the atriopore; they were first served upon the gut and diverticulum by van Wijhe (1913).

Particular attention has been paid to the system of cells that lies upon the t and its diverticulum, which has been studied in detail by Boeke (1935 *a*, *b*), rtisinghe (1940), and Holmes (1953). These authors have reached different nclusions as to the status and synaptic connexions of the cells.

The nerve-fibres that are in connexion with this system of nerve-cells are ned with the central nervous system through the dorsal-root nerves. The angement of the three rami of the ventral branches of the dorsal-root rves has been previously described; it is illustrated in fig. 1. The ascending ceral rami run up the parietal wall of the atrium and are then connected th the system of nerve-cells upon gut and diverticulum. In the pharyngeal gion the nerves run upon the denticulate ligament; farther back they join

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directly with the roof of the gut. The location of the cell-bodies which giv rise to the fibres composing the ascending visceral rami is in doubt.

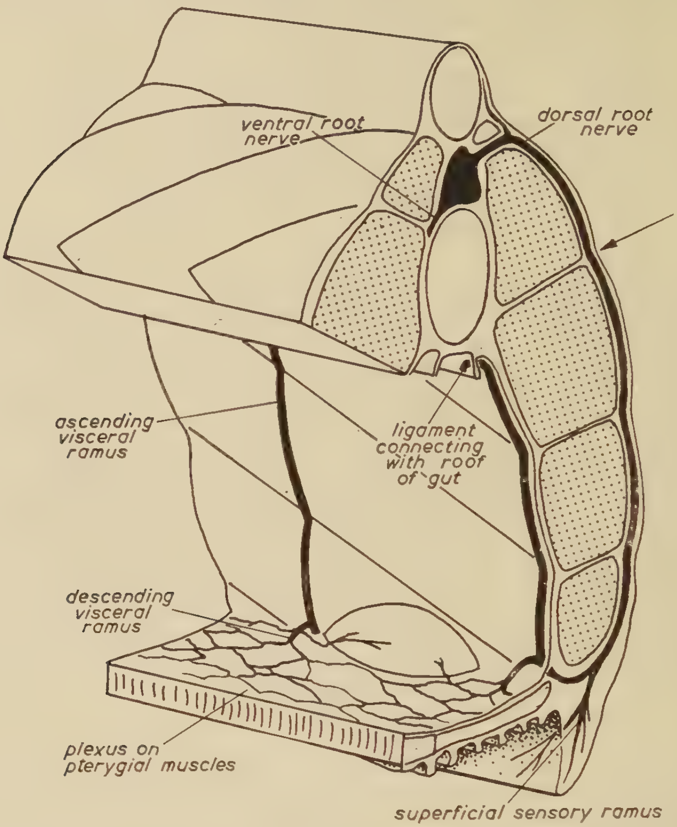
ventral root nerve
dorsal root nerve
ascending visceral ramus
ligament connecting with roof of gut
descending visceral ramus
plexus on pterygial muscles
superficial sensory ramus

Fig. 1. Diagram illustrating the relations of the branches of the dorsal-root nerve. In the experimental series of animals the nerve was interrupted at the position indicated by the arrow.

van Wijhe supposed that the atrial nerve-cells sent their axons up into th spinal cord (thus, that the ascending visceral rami contain centripetal fibres He stated, 'I have discovered under the atrial epithelium that covers the live intestine and gut an enormously large number of splendid multipolar cell whose axis cylinder runs along the dorsal nerve roots to the spinal cor (van Wijhe, 1913). He regarded the cells as sensory, yet spoke of them a 'sympathetic' cells. Both Boeke and Kirtisinghe supposed that central cell sent axons out to the atrial nerve-cells (thus, that the ascending visceral ram contain centrifugal fibres), and that these axons synapsed peripherally wit the atrial cells, in a manner analogous with the synapses of the vagal fibres o vertebrates upon the cells of the enteric plexuses. Holmes returned to va Wijhe's view that the cells were joined with the central nervous system b eir own axons; he pointed out that this view implies that the character and lations of the atrial cells of amphioxus are very different to those of the teric cells of the vertebrates. These opposing views are illustrated in fig. 2, here the arrangement of the vagal enteric fibres of the vertebrate is shown r comparison.

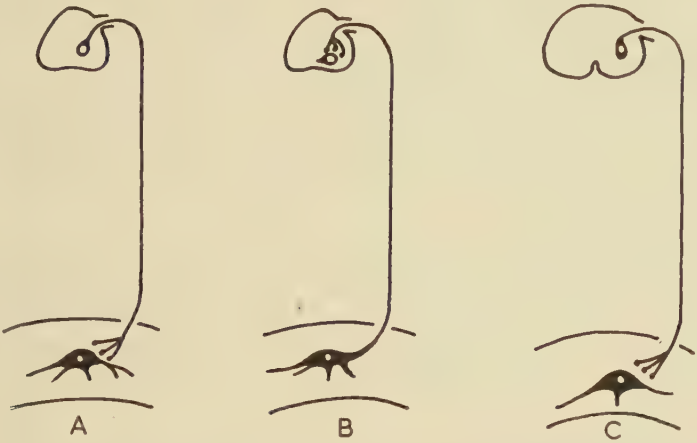

FIG. 2. The alternative views of the connexion of the atrial nerve-cells with the central nervous system. A represents the view held by Boeke and Kirtisinghe. B that held by van Wijhe and Holmes and confirmed in the present paper. C illustrates the connexion of a vertebrate vagal fibre with an enteric cell.

The interconnexion of the cells at the periphery is also the subject of spute. Boeke concluded that the cells are joined together asynaptically, verywhere lying in a syncytial arrangement'. Neither Kirtisinghe nor olmes was able to confirm this view. Holmes suggested that the dendrites the cells were more often terminal structures than the sites of synaptic nnexion between neighbouring nerve-cells.

The present investigation was undertaken in an attempt to resolve these ntradictions, and to answer the further question of the origin of this system cells in ontogeny.

MATERIAL AND METHODS

Adult amphioxus were obtained from the site in the Bay of Naples near the lla Galotti, and fixed (after menthol anaesthesia) in sea-water Bouin, 10% rmalin, mercuric-formalin, and Helly. Whole mounts and paraffin and llodion sections were stained by Holmes's method (Holmes, 1947), by the elschowsky method, and by its variant used by Kirtisinghe (Hill, 1927). ne observations described in this paper are largely the result of the examination of whole mounts stained with Holmes's technique.

Since previous authors had interpreted the results of the different staining ethods that they had used in a different manner, all the methods previously nployed were used, in order to discover whether the different procedures gave significantly different results. It was found that the pictures given by the Bielschowsky methods did not differ from that given by Holmes's method both of the procedures give somewhat different results after different methods of fixation. Different methods of fixation are an important variable, not only in the quality of the impregnation obtained, but also in the general appearance of the cells; it seems that sea-water Bouin or Zenker-formalin is the mos satisfactory fixative (it is less easy to stain the cells after the latter fixative).

In addition to the adult amphioxus material, specimens of very young amphioxus (5 mm post-metamorphic, adolescents 15–20 mm long) were staine as whole mounts, as were also several specimens of adult *Asymmetron*. All this material had been fixed in sea-water Bouin and stored in 90% alcoho since 1931; successful impregnations were obtained by a modification o Holmes's technique.

A series of degeneration experiments was also carried out in order to trac fibre paths in the atrial system. Dorsal-root nerves were interrupted by cauter with a needle, and the animals fixed at varying times after this operation. Two to five nerves were interrupted upon one side in each specimen, at the position indicated upon fig. 1. The animals lived well after the operation, in jars unde aeration, in a constant temperature room at 18° C; they were kept alive up t 30 days after the operation.

Serial paraffin and collodion sections of the operated animals were staine with Holmes's technique (the classical staining methods for degeneratin myelin cannot be used, as the nerves of amphioxus are 'non-medullated').

Results

The connexion of the atrial system with the central nervous system

The atrial system of cells on the gut and gill bars is joined with the centra nervous system by the dorsal root-nerves in the way shown in fig. 1. Experimental interruption of these nerves, followed by histological examination o the operated animals fixed after varying periods, will therefore show (by th position of the degenerated fibres) whether or not there are axons of peripheral cells running into the central nervous system in the nerves. If centra degeneration takes place after the operation, then this means that the nerve do contain such axons, and that the atrial system joins with the cord by th axons of its own peripheral cells, and not by fibres of central cells passing ou along the nerves to synapse with the atrial cells peripherally.

The degeneration experiments, which were carried out in the manne described earlier, gave clear indications of central degeneration, after sectio of the dorsal root-nerves of the atrial region. In animals fixed between 15 an 27 days after the operation, there are evident signs of late stage Walleria degeneration of many of the fibres in the spinal cord.

The degenerated fibres were fragmented into short irregular pieces whic stained deeply with silver. These were more or less swollen and appeared a chains of small thickened pieces of axoplasm. These appearances are seen i

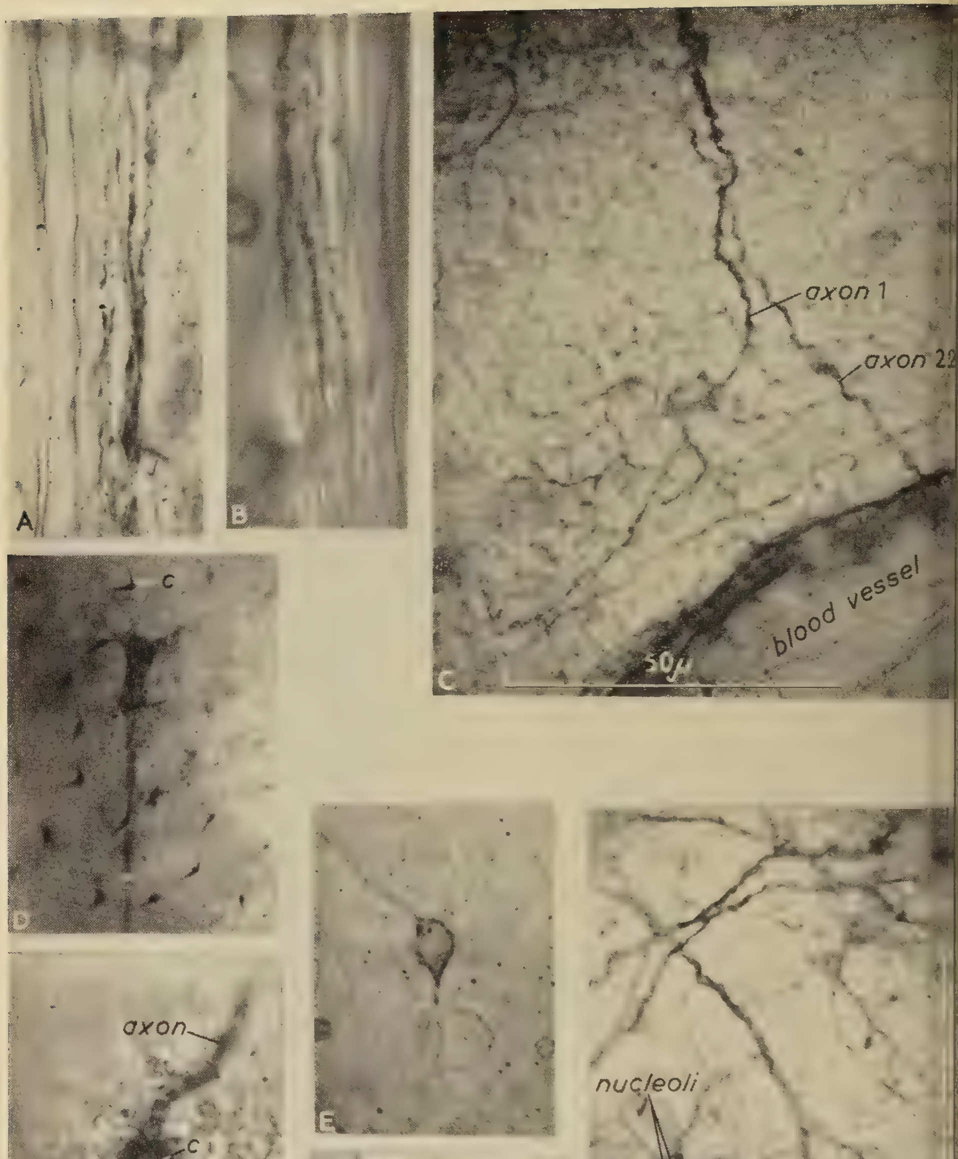

Fig. 3

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ngitudinal sagittal sections of the spinal cord of operated animals (fig. 3, A, B). is certain that these are not artifact, for they are not seen in normal animals ed and stained in the same way, and further, in the operated animals rmal and degenerated fibres can be seen running side by side.

The endings of the degenerated fibres have not been determined (the imal fixed soonest after the operation already showed advanced degenera- on of the axons, and it is likely therefore that the optimum time for observa- on of the endings had passed by this time); it seemed in some cases that ey ended as pericellular baskets upon cells of medium size.

The number of degenerated fibres in the cord, after section of only two rsal-root nerves, is remarkable, as also is the distance that these fibres run and down in the cord; it seems that they are in connexion with much of e first three-quarters of the cord, and that in each dorsal root-nerve in the rial region there must be a large number of axons of peripheral cells.

The results obtained from these degeneration experiments show that the rsal-root nerves of the atrial region contain many axons of peripheral nerve- lls. Now, the only peripheral cells in the region studied (the pre-atrioporal gion) are those of the atrial plexus, the cells lying upon the gut, gill bars, and rietal walls of the atrium.

These cells send their axons to the ascending visceral rami in each segment, hich then joins with the other two ventral rami, and makes up the dorsal- ot nerve (fig. 1). It is not possible to cut the ascending ventral ramus (as it s inside the atrium), but it may be shown that it is only in this ramus that ere are axons of peripheral cells, so that it is quite certain that it is the cells the atrial plexus that are sending their axons up into the central nervous stem. Of the other two rami, the superficial one to the skin contains only nsory fibres, which end freely amongst the epidermal cells of the ventral rt of the body; the descending visceral ramus contains only motor-fibres to e pterygial muscle forming the floor of the atrium; so it is clear that the ons which degenerate must begin in the ascending visceral ramus.

After section of the dorsal-root nerve, no changes can be seen in the cells

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FIG. 3 (plate). A, degenerated fibres in the spinal cord. Sagittal section at $25\,\mu$; Bouin, olmes's silver, paraffin.

B, similar field from another operated animal. Sagittal section at $30\,\mu$; Bouin, collodion, olmes's silver.

C, isolated neurone on mid-gut of adolescent individual, showing extent of dendritic field, d axon leading up to ascending visceral ramus. Whole mount, Bouin, Holmes's silver.

D, neurone on mid-gut of *Asymmetron*. Note 'double' cilia (some in focus above the axon) the atrial epithelial cells. Whole mount, Bouin, Holmes's silver. *c*, cilia of atrial epithelial lls.

E, neurone on mid-gut of *Asymmetron*. Bouin, whole mount, Holmes's silver.

F, neurone on mid-gut of 7-mm individual. Atrial epithelial cells and their cilia (a cilium in focus above the nucleus of the neurone); wide axonal process. Whole mount, Bouin, olmes's silver. *c*, cilium of atrial epithelial cell.

G, similar field from another individual. Few dendritic processes (as compared with C from adolescent), wide axonal process. Whole mount, Bouin, Holmes's silver.

H, neurone pair from diverticulum of adolescent. Note two nucleoli of one cell, and close nnexion between cells. Whole mount, Bouin, Holmes's silver.

of the atrial plexus, nor in the ascending visceral rami, although the superficial sensory rami are entirely composed of degenerated fibres. It is ver difficult to give a quantitative estimate of the number of degenerated fibre upon the central side of a sectioned dorsal root nerve because at the leve where the cuts were made, the dorsal root-nerve almost immediately turn inward below the skin, and cannot be clearly followed in section. It is also th case, as will be seen below, that any such estimate after degeneration experiments upon cold-blooded animals needs to be interpreted with great caution It may be suggested, however, that there is no impossibility, on the score o the number of degenerated fibres visible, in the whole of the system of cells i the atrial plexus being joined to the central nervous system by their axons.

It is concluded, therefore, that the cells of the atrial system are connecte with the central nervous system by their axons, which rise in the dorsal-roo nerves. This conclusion is supported by the appearance of whole mounts o the gut and diverticulum, in which it is clear that the nerve-cells are sendin their axons up into the ascending visceral rami; this is perhaps most eviden in younger animals where there are relatively fewer cells in the system (fig. 3 F, G). Whole mounts of *Asymmetron* material have a very similar arrangement and it is likely that the system is essentially the same as that in amphioxus.

It is interesting that in cold-blooded animals degeneration of medullate fibres depends very markedly upon the temperature at which the animals ar kept after the operation. Not only does degeneration proceed more slowl at a lower temperature, but it may manifest itself in a strikingly erratic manner which will make interpretation of the experiments difficult, as Gamble Goldby, and Smith have recently shown (1957). In my own experiments a Naples, the operated animals were kept at 18°–20° C in the constant temperature room; other unoperated animals were kept in the laboratory circulatio at a temperature slightly higher than this, and it is probable that in the se the temperature may even rise to about 22° C in the middle of the summer Thus the degeneration was allowed to proceed at a temperature slightly belo the maximum which the animals would tolerate. In similar experiment carried out at Plymouth, Holmes was unable to perceive signs of degeneratio after 5 days; his operated animals were kept at the temperature of the circulating sea-water, which was about 15° C in summer. It was because Holme had seen no sign of degeneration after 5 days that no animals were fixed i the present series of experiments earlier than 15 days; from the advance state of degeneration at this time, it seems likely that the first stages o degeneration would have been visible at about 5–7 days.

The results obtained by the authors cited above in their work on degeneration in *Lacerta* show that it is possible that no visible signs of degeneratio may occur (although axons have certainly been interrupted) up to 3 week after the section of the nerve; this depends entirely upon the temperature For this reason, it is difficult to give reliable quantitative estimates of th numbers of axons of peripheral cells within the dorsal-root nerves. It may b also that the sensitiveness of cold-blooded animals to variations in temperatur

this respect accounts for the different rates of degeneration that evidently
ccurred in animals kept at 15° C and 18°–20° C.

The conclusion that the atrial cells are connected to the central nervous
stem with their own axons, as van Wijhe and Holmes had earlier suggested,
of great interest when the function and homology of the system are con-
dered. It is clear that the system is very different from that of any vertebrate,
nd it seems that it must function in a different way. These problems will be
nsidered in a later section of this paper; I wish to emphasize here that the
stem of atrial nerve-cells in amphioxus cannot be compared in any obvious
anner with the sympathetic or parasympathetic components of the autono-
ic systems of higher vertebrates; the essential difference between them is
ustrated in fig. 2 (p. 245).

he form and interconnexion of the nerve-cells in the atrial plexus

The account of the atrial nerve-cells given below is based almost entirely
pon the examination of whole mounts of the diverticulum and fore-gut,
repared from adult specimens of amphioxus. It is this part of the system
at is easiest to study, and it is this part that has been most studied by pre-
ous workers. In this region of the atrial system (unlike that part of the
stem upon the gill bars, or upon the parietal wall of the atrium), the nerve
lexus can be readily observed in whole mounts without dissection of under-
ing tissues; moreover, the system is here essentially two-dimensional, and
o offers exceptional opportunity for the examination of cell shape and con-
exion. In this respect, the atrial system of the gut of amphioxus is unlike
at of the enteric systems of craniates, where not only are the underlying
ssues more opaque after silver-staining, but also the nerve-cells of the
lexuses are found at different depths in the preparation and connexions
etween them cannot be so easily determined.

The hind-gut is not so suitable for study in amphioxus, for melanophores
und there interfere with the silver impregnation of the nerve elements;
me preparations of this part of the system in both amphioxus and *Asym-*
etron have shown that the system there is very similar to that in the anterior
arts of the gut.

In *Asymmetron* and in young specimens of amphioxus, the arrangement of
e atrial system is essentially the same as in the adult amphioxus; there are
ifference in detail, which will be discussed in the course of this section.

The neurones under the atrial epithelium covering the gut and diverticulum
re extremely abundant; in an adult there are at least 6,000 cells upon the
iverticulum alone; that is, in an area approximately 39 mm square, there are
,000 cells. This concentration is normal throughout the system upon the gut
f the adult; upon the whole of the gut, therefore, there may perhaps be
2–15,000 nerve-cells. In transverse section there are more cells visible in
e atrial system than there are in an equivalent portion of the spinal cord.
t is not surprising, therefore, that there are many degenerated fibres running
the cord after section of a single dorsal root-nerve.

Each of the nerve-cells possesses several processes. These dendrites very soon branch, and the whole area around the cell becomes covered by the processes from it (fig. 3, C). The same kind of processes as are given off from the cell-body, are found along the course of the axons, even at considerable distance from the cell-body, as can be seen in fig. 4, A. In this way, there are processes from nerve-cells, or cell-bodies themselves, over most of the area of the gut; under oil, in fully impregnated whole mounts of the gut, it is not possible to find any field which does not contain nerve-fibres.

These cells and fibres lie just underneath the layer of ciliated atrial epithelium which covers the gut; this can be clearly seen in fig. 3, D, F, where the cilia of the atrial cells are in focus above the nerve-cell. It is interesting that the cilia of the amphioxus atrial cells seem to be borne singly, whereas in *Asymmetron* (fig. 3, D), it seems that the cilia are always paired, each cell bearing a double cilium. When pieces of atrial epithelium are dissected off the gut and impregnated separately, the nervous plexus is always found with its finest processes; so it appears that the plexus is closely applied to the atrial epithelium, and that the processes of the nerve-cells do not run to the underlying cells of the gut.

In certain cases also, it seems that the dendrites and axon processes are related to the boundaries of the cells of the atrial epithelium; it may be that these processes are actually running between the epithelial cells. Such appearances are seen in fig. 4, A, where the nerve processes outline polygonal shapes characteristic of the epithelial cell boundaries (the epithelial cells are seen in focus in fig. 3, F). However, in other cases, as in fig. 3, C, this relation does not occur, and the nerve processes pass across the epithelial cell boundaries.

Previous authors have supposed that the endings of the nerve processes lie at a different level from the nerve-cell body, passing up and down out of the plane of the atrial epithelium; if this is so, such endings are beyond the resolution of the light microscope.

The nerve-cells vary a good deal in size and shape. This may most simply be seen by comparing the figures; it is evident that the cells of the young amphioxus plexus are in general smaller than those of the adult amphioxus as also are the cells in *Asymmetron*. The form of the cells varies greatly after different methods of fixation; it has been suggested above that those animals fixed in Bouin show the nearest approximation to the condition in life, a con-

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FIG. 4 (plate). A, two cells and their processes from the diverticulum of an adolescent individual. Note interaxonic processes. The larger fibres in the field are all the axons of nerve-cells, two of which are seen. Whole mount, Bouin, Holmes's silver.

B, closely associated cells from diverticulum of adult. Whole mount, mercuric-formalin, Holmes's silver, gold-toned.

C, associated cells from mid-gut of adolescent. Whole mount, Bouin, Holmes's silver.

D, monaxonic (?) binucleate cell from diverticulum of adult. Whole mount, mercuric-formalin, Holmes's silver, gold-toned.

E, biaxonic cell pair. Diverticulum of adult, whole mount, mercuric-formalin, Holmes's silver, gold-toned.

F, junctional appearance at axon crossing. Adult, whole mount, Bouin, Holmes's silver, gold-toned.

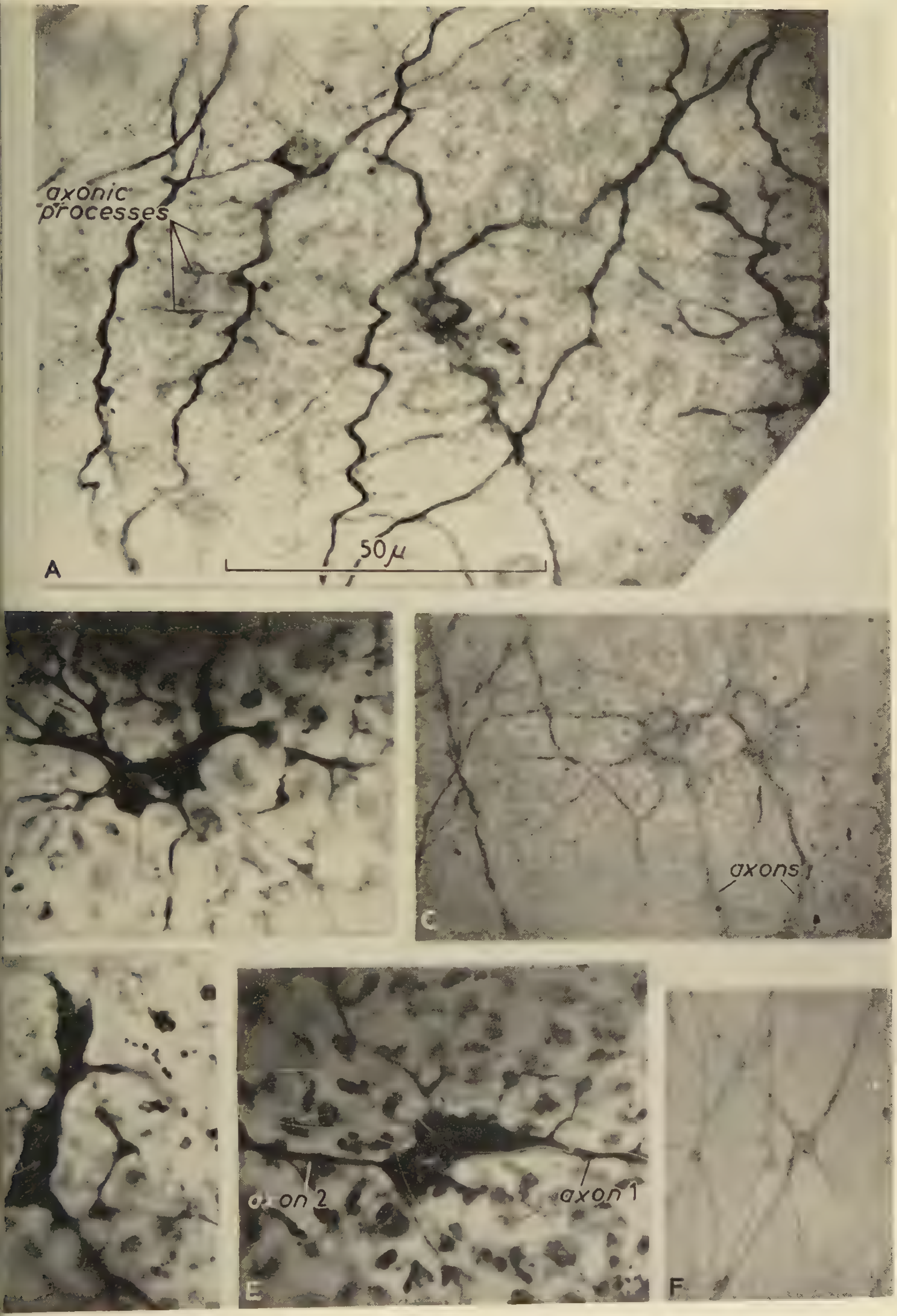

FIG. 4

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usion supported by some observations that were made upon the system in e (by phase contrast). Phase contrast observations upon the system are not ry satisfactory, since, in the living animal, it is a very difficult matter to ssect the atrial epithelium (and thus the nerve plexus) away from the undering opaque intestinal epithelium.

Apart from fixation differences,the form of the neurones seems to depend rgely upon their relation with the other cells and fibres in the system. Where her cells are sparse, the cell-body is more or less oval; processes radiate om it in a symmetrical manner. Such cells are seen in fig. 3, C, F. Where ere are many other cells in the neighbourhood, their processes are related each other (most if not all of the neighbouring cells are in connexion), and e form of the cell-body and arrangement of the processes are less symetrical, as in fig. 4, A.

All the cells are multipolar, with up to 7 dendrites; these usually proceed om expansions of the cell-body. The dendrites branch irregularly as soon as ey leave the cell-body; where the cell is relatively isolated, this branching ay be almost diagrammatically dichotomous.

Occasionally, what appear to be bipolar cells can be seen in the plexus. oth Boeke and Kirtisinghe recognized such cells as a second, distinct cell pe, but it seems more reasonable to suppose that they do not represent a cond type of cell; rather, they are either cases where one axon runs over nother cell-body, or else cells which are in the process of establishing themlves in the plexus; this hypothesis will be examined below (p. 257). It is ggested that they are best regarded as cells which are in the process of tablishing themselves in the plexus.

In almost all cases there is not a sharp distinction between the cell-body self and the beginning of its axon: the axon itself gives off processes which e in size and relations similar to the dendrites. These processes of the axon ay be given off even at considerable distance from the cell-body, as may be en in fig. 4, A. The axon is usually clearly recognizable from the other proesses of the cell (despite Boeke's contention that 'a difference between eurite and dendrite was not to be found'). It is thicker, and (in silvernpregnated material) often lighter in colour than the other processes. This especially clear in young animals (where there are fewer cells in the plexus), s in fig. 3, G, from a 5-mm individual. Such appearances leave no doubt that e cells are actually sending their axons up into the ascending visceral rami. hese axons collect into bundles upon the top of the gut and diverticulum; e bundles leave in a more or less segmental manner and enter the ascending isceral rami. In the adult, but not in the young animal, there are few cells long the course of these axon bundles towards the top of the gut; the majority f the cells lie towards the ventral parts of gut and diverticulum. It is interesting, hen the origin of the system is considered, that the axons of the nerve-cells pon the diverticulum do not run backward to join the ascending visceral rami t the level of the origin of the diverticulum, but rise to the dorsal part of the iverticulum itself, and leave through the gill bars and denticulate ligament.

Holmes stated that the processes of the cells ended in general freely: 'i seems that these dendrites are more often terminal structures than the site o synaptic connections between neighbouring nerve cells.' Some of the dendrite are undoubtedly terminal structures. One such is seen in fig. 5, D (opposit p. 255), where it ends freely in a small enlargement (in the same plane as th cell-body). Even Boeke, who supposed that the cells were anastomose together, figured some of the processes as ending freely.

It seems, however, that in the majority of cases, the dendrites and axoni processes are in connexion with neighbouring cells and their processes. Th nature of the connexion is such that it gives the appearance of complet anastamosis. Where fine filaments meet or cross, there are often (though no always) triangular junctional areas, and these are even seen when two axon are crossing each other at right angles (fig. 4, F). Similarly, when a proces ends upon a neighbouring cell, there is also a flattened junctional area.

Although these appearances are very suggestive of asynaptic continuity o the cells within the system, it is perhaps most reasonable to regard them as th natural consequence of the fluid properties of the nervous material; they need not necessarily imply anastamosis. Pantin (1952) has convincingly show for a similar type of junction in the Anthozoa that these appearances do no necessarily mean that adjacent fibres have fused at the points of junction However, as will be seen below, the possibility of asynaptic connexion cannot be completely excluded, and, indeed, it is suggested upon othe grounds.

In a general way, the arrangement of the atrial plexus upon the gut and diverticulum of *Asymmetron* is the same as that described above for amphioxus The cells of the plexus are smaller (fig. 4, D, E), and also it seems that they are much less densely placed upon the gut. Their processes usually procee from the cell-body alone; axonal processes are apparently rare, and this give the plexus a much more 'open' appearance. It may be that this difference i due to incomplete impregnation of this old material, but it seems that on th whole the *Asymmetron* plexus is comparable to that of the young amphioxu rather than to the plexus of the adult amphioxus described above. This i interesting when the development of the two families is considered. Th larvae of *Asymmetron* (the amphioxides larvae) are relatively greatly retarde with respect to the amphioxus larvae; perhaps it may be correct to say tha the whole sequence of development in *Asymmetron* is performed at a slowe rate than in amphioxus, so that in the adult *Asymmetron*, characters similar t those of the young amphioxus are found, rather than the comparison bein between the two adults.

The asymmetry of the gonads results in a different mode of origin of th ascending visceral rami from the common visceral rami, on the two sides o the atrium; there is a much greater innervation of the walls of the gonad sac than in amphioxus, and the atrial papillae are arranged differently; but, otherwise, the atrial system is like that of amphioxus. The details of these differences will be described in a subsequent paper.

he problem of the multinucleate cells in the system

In the atrial nervous system, as in the autonomic systems of vertebrates, ere are occasionally found cells with two or more nuclei. Such a cell was gured by Kirtisinghe (his text-fig. 1). These cells are not common in the stem; they may perhaps make up 0·5% of the whole. It seems that they are mmoner in young than in old animals, though they may be found even in ature animals. An extensive literature exists upon such cells in the nervous stem of vertebrates; there are various views as to their significance. It has en suggested that they are pathological, but it seems that in most cases this not so and that they are in fact usual components of the system (Hogue, 52). Two views are then possible. Cole (1925) suggested that in the myenteric exus of the frog, multinucleate cells were formed by the fusion of cells hich were originally separate. On the other hand, it has been supposed that ultinucleate cells are the result of nuclear division unaccompanied by cytoasmic division. Different authors have supposed that this nuclear division ay be mitotic or amitotic. In tissue culture of nerve-cells derived from uman foetuses, Hogue has shown convincingly that the last alternative takes ace and she figures stages in this process.

In amphioxus, however, it seems that the first suggestion, that originally parate cells later fuse, may also be correct. Figs. 3, H and 4, B, C, E show lls that are in such intimate connexion that they appear to be joined by a ytoplasmic veil'. Each cell possesses its own axon, which (in fig. 4, E) can seen running in the opposite direction to the axon of the other cell. The eil' connecting these cells seems to be composed of the nerve-cell cytoplasm, nd is not part of a sheath-cell system; indeed, remarkably enough, sheathlls appear to be absent from the atrial system. This will be discussed below, hen the general question of the sheath-cells in the peripheral nervous system considered.

Yet this way in which the cells may become intimately connected can carcely explain other appearances that are found in the atrial system. If it ere the case that the binucleate cells were formed in the same way as the lls which are connected intimately as described above, then it would be ifficult to explain why they possess only one axon. In the vertebrate plexuses, he cells synapse at the periphery with extrinsic fibres. Thus it is not surrising that they can fuse as Cole suggested; but in the atrial system of mphioxus, the cells are sending their axons up into the central nervous ystem, so that as the binucleate cells are not bi-axonic the cells shown in g. 4, B, C, E cannot represent the first stage in the formation of monaxonic i- or multinucleate cells.

Fig. 4, D shows such a binucleate cell; the appearance suggests nuclear ivision without cytoplasmic division. That the axons of the amphioxus ystem are arranged differently from those of the vertebrate enteric cells ould seem to preclude that these binucleate cells can later divide, giving ise to appearances such as fig. 4, B, C, E. For, if the cells divide partially or totally, it is difficult to see in what way they may become connected with the central nervous system; it does not appear likely that they can grow new axons up into the dorsal roots. When the origin of the system in ontogeny is considered, this appears even less likely (for it will be seen below that the atrial cells most probably migrate out down the dorsal root-nerves from the central nervous system), so that it is not suggested that the atrial cells increase in number within the system.

It is here supposed that the multinuclear cells, and the cells which are closely associated, arise in a different way and that both of the processes which have been suggested as giving rise to multinucleate cells very probably occur in the atrial system. The first alternative implies that some at least of the cells in the system are in asynaptic connexion; this condition is not at variance with the suggested function of the system (p. 259).

Sheath-cells in amphioxus

In connexion with the hypothesis of asynaptic continuity in the atrial system and the fusion of initially separate nerve-cells, it is interesting to consider the arrangement of the sheath-cells which may surround the nerve-tissue. Along the course of the dorsal root-nerves, as they pass just under the cutis, there are a number of nuclei. Similar nuclei are seen along the course of the bundles of fibres emerging from the ventral roots, and passing amongst the myotomes. Those along the dorsal root-nerve are seen in fig. 5, A.

A good deal of confusion exists in the literature about these nuclei. Hatschek (1892) figured what appear to be these nuclei along the course of the dorsal root-nerve as it passes out between the myotomes towards the skin; he supposed them to be the nuclei of nerve-cells, and this hypothesis has been widely supported, despite the fact that Heymans and van der Stricht (1898) saw no trace of nuclei in this position. Krause (quoted by Young, 1950) figures nuclei along the course of the dorsal-root nerves, which he supposed to belong to nerve-cells; again, Johnston stated that there are cells along the beginnings of the dorsal root-nerves (1905), and these, he imagined, represented the dorsal root ganglion-cells which were more diffuse than in the vertebrates.

These hypotheses I believe to be completely erroneous. It is certain that these are not the nuclei of nerve-cells: in fact, they are the nuclei of the epineurium which surrounds the nerve as it passes along under the surface of the skin. They lie upon the main nerve-trunks and along the branches that

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FIG. 5 (plate). A, dorsal-root nerve under skin of lateral body-wall, showing sensory branch to skin, and cells of the epineurium. Whole mount, Glee's Bielschowsky.

B, neurones from mid-gut of adolescent, showing passage of the axon of one over the other (leading to the spurious appearance of bipolar cells). Whole mount, Bouin, Holmes's silver.

C, cell-bodies in plexus upon denticulate ligament in adolescent individual. Note absence of dendrites. Whole mount, Bouin, Holmes's silver.

D, E, F, cell-bodies along the course of the ascending visceral rami in young (5–7 mm) individuals. Whole mounts, Bouin, Holmes's silver.

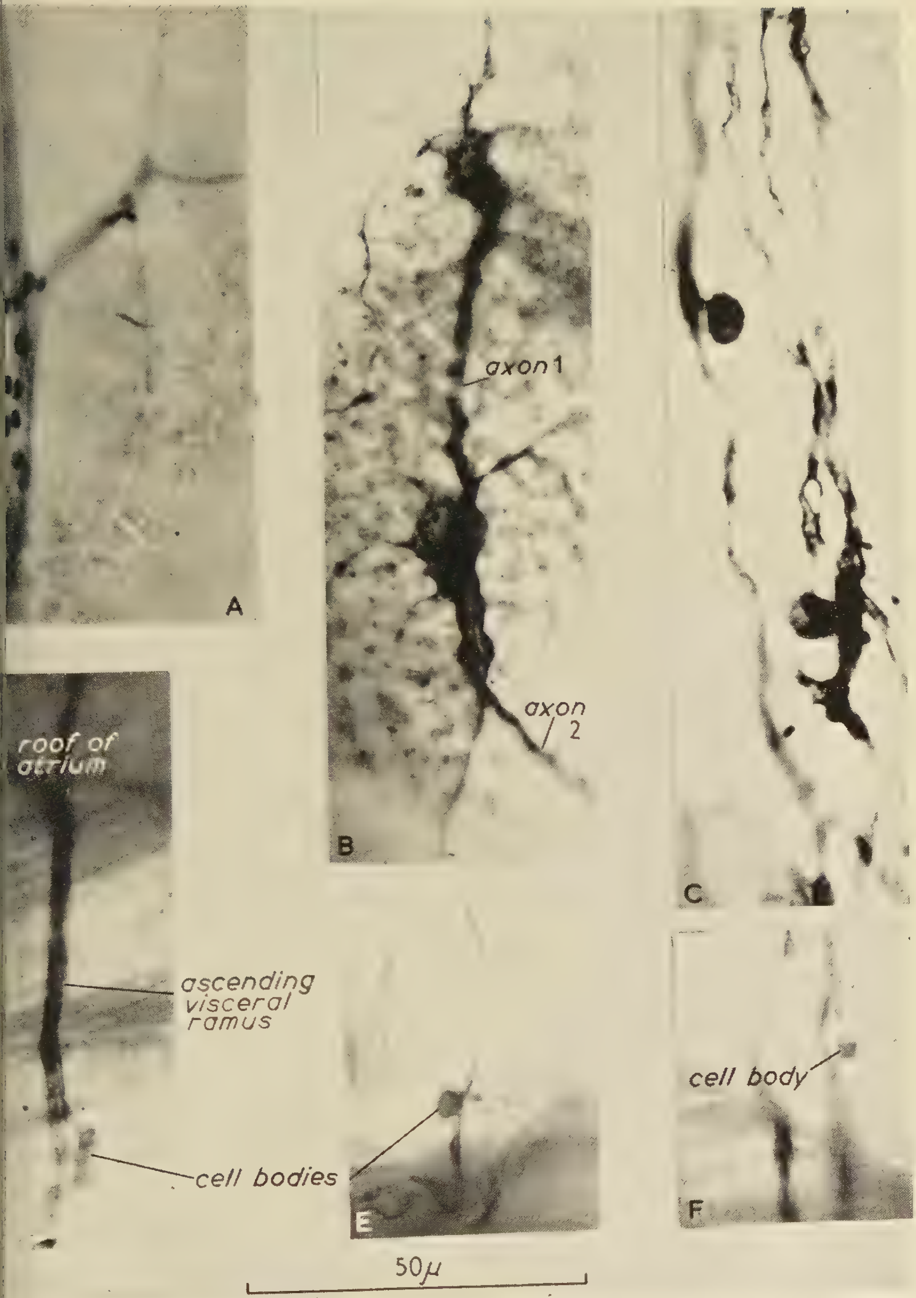
A
axon 1
axon 2
B
C
roof of atrium
ascending visceral ramus
cell bodies
E
cell body
F
50μ

Fig. 5

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given off from them (sensory to the skin), as can be seen in fig. 5, A. It is obable that they are derived in ontogeny from the dendritic connective sue-cells which lie everywhere in the cutis; for such cells are in the larvae sociated with the branches of the rostral nerves. As the nerves grow out, ese cells come to form a connective tissue sheath around them.

No nuclei of any kind are found along the course of the nerve-fibres of the ial system, even when the nuclei of the atrial epithelial cells, their cilia, and e finest processes of the nerve-cells have been impregnated. Similar techques stain the nuclei of Schwann cells in vertebrates. It seems that the rangement of the sheath around the nerve-fibres is different from that found vertebrates. It is hoped soon to examine this problem with the aid of the ectron microscope.

he origin of the atrial system

The early origins of the atrial system are as yet unknown. I have not been le to obtain material suitable for nerve impregnation at an earlier stage than at of the very young post-metamorphic individuals (between 5 and 7 mm ng), where there are about 12 pairs of gill slits and the atrium is already in adult condition. At this stage, the atrial system is essentially the same as it in the adult, except that there are many fewer cells upon the gut and divertiulum (the diverticulum is just beginning to grow out). Fig. 6 shows the rangement of the system in such a larva.

The nerve-cells are superficially similar to those of the adult plexus, but ey differ in that the cell-body is often slightly larger than that of older imals (though smaller than that of the adult) and the processes from it are wer and simpler. Cells from the plexus of these young animals are seen in g. 4, F, G, to the same scale as those of the other figures.

The axon of the cell is more distinct from the other processes than in the der animals, partly because it is very broad for a long distance from the ell-body, and also because there are fewer fibres in the system. There is much ss variation in cell size than in the adult. Almost all of the cells in the very oung animals are similar to those illustrated; this size is slightly greater than at of the young adolescents (15–20 mm long), which were the next stage xamined. The relatively larger size of the cell-body in young animals may ost probably be attributed to the fact that the cell processes have not yet ully developed.

The nuclei of the cells in the younger animals are very distinct, their ucleoli stain heavily with silver. About 20% of the cells possess nuclei with wo nucleoli, a proportion much greater than in the adult, where, although ells are found containing two nucleoli, these are only at about 1% of the hole. It is difficult to say what the significance of this observation may be.

It is known that a proportion of the nuclei in animals where the usual ucleolar number is two or more have but one nucleolus. This may be most asily explained by the hypothesis that at a cell-division, the nucleolar rganizing regions of the chromosome pair lie close together, and the resultant large single nucleolus is formed by the fusion of the two found in other cells In amphioxus, cells other than the nerve-cells possess two nucleoli, but mor often there is only a single nucleolus.

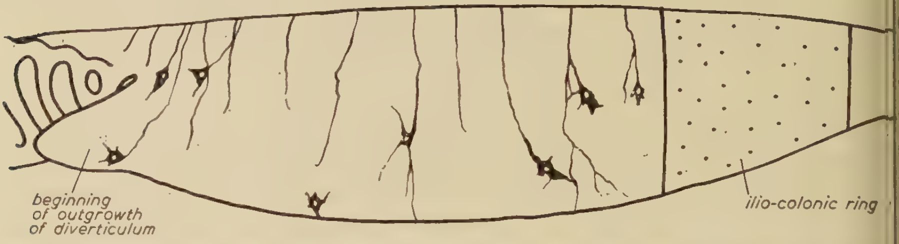

FIG. 6. Diagram to show the approximate number and size of the nerve-cells upon the gut o a young (5-mm) individual.

Since the atrial epithelium is fully differentiated at the stage of the younges individuals examined, and since there are relatively few cells in the plexus, i is evident that the greater proportion of the cells of the adult plexus have no yet appeared in it at this stage, and also that they do not arise later by th differentiation of the ectodermal atrial epithelial cells. They must therefor arise either by the division of the cells in the young animals, or else they mus migrate into the system from elsewhere.

If cell-division in the young animals accounted for the increase in cell-number in the plexus (and also for the occurrence of closely associated an binucleate cells), then it would be expected that stages in this process migh be often observed in the young animals.

However, this is not the case, and it is therefore concluded that division o the atrial cells does not play a significant role in the increase of the number o atrial cells during adolescence. In these small amphioxus, which were examine as whole mounts after dissection under a binocular, it was only feasible t observe the cells upon the gut and diverticulum, and upon the parietal wal of the atrium, so that it is possible (but somewhat improbable) that divisio of the cells may take place whilst they are upon the denticulate ligament, an that they then move down on to the gut.

It is much more probable, however, that the atrial system is formed, and added to, by the emigration of cells from the central nervous system, along the dorsal root-nerves. It has long been known that the enteric cells of vertebrates (parasympathetic) migrate out along the visceral branches of the vagus (Kuntz, 1909) in the course of development; various lines of evidence point to an analogous process taking place in amphioxus.

The corpuscles of de Quatrefages (in the adult these are found at the ends of the branches of the rostral nerves) are known to migrate out from the brain along the rostral nerves, as Franz (1927) suggested, and as I have observed in the amphioxides larva of *Paramphioxus*. Johnston (1905) found that in horizontal sections of the nerve-cord, nerve-fibres running longitudinally tended to bend outward to the dorsal roots as they ran past them, suggesting that vement of material had taken place out from the cord. More direct evidence provided by the conditions in the postmetamorphic individuals that are cussed above.

Fig. 5, D, E, F illustrates what may most probably be interpreted as nerve-l bodies along the course of the ascending visceral rami, where these rami e ascending the parietal wall of the atrium. In 20 individuals, which were ll impregnated in this region, such cell-bodies were only observed upon e course of 7 of the ascending visceral rami. They were all found in the ne position where the ramus begins to ascend the parietal wall, just after has separated from the descending ramus running to the pterygial muscles g. 1). None was found in any other position along the dorsal-root nerve anches and none in any position in older animals. If, then, these are in fact be interpreted as cell-bodies in the process of emigration from the spinal rd, it is clear that the greater part of the cells of the plexus must have passed e point where these cells were observed, before the stage of development ained by these animals.

Perhaps it may be that they emigrate during the metamorphosis, whilst the rium is being formed, i.e. just before the stages which I have been able to tain. If this conclusion is correct, a further point in the process may be ferred. Since the greater part of the cells have passed the ascending visceral mi by this stage, and since there are relatively few cells upon the gut, it is obable that the great proportion of the cells of the adult plexus are at this ge found upon the denticulate ligament or at the roof of the atrium. From is position they migrate yet farther as development continues, and enter e plexus upon the gut.

This inference is supported by two observations. The first is that there are any cells upon the denticulate ligament in young animals, and that these lls do not possess dendritic processes, but have rounded cell-bodies which at the side of the fibres, in the way illustrated in fig. 5, C. Even in adult imals, it seems that cells are still entering the plexus upon the gut from is 'sump' of cells in the roof of the atrium, for nuclei are found upon the on bundles leaving the roof of the gut, again without dendritic processes. has already been stated (p. 251) that it is most probable that 'bipolar' cells e those in the process of establishing themselves in the plexus; they are ost often seen on the roof of the gut, and may most simply be interpreted cells which have not yet developed all their dendrites. It is also the case at many of the bipolar cells figured by Boeke (for instance, his text-fig. 7, 35*a*) are actually cells with one axon, but an axon of another cell runs over em, as in fig. 5, B.

Secondly, there is a very striking increase in the number of nerve-cells ithin the plexus upon the gut, in later stages of development: in animals mm long the cells are as dense as in the adult plexus. The majority of these lls lie towards the top of the gut and diverticulum, and there are many cell-odies along the course of the collecting bundles of axons at the top of the stem. It has been pointed out (p. 251) that the axons of the cells upon the diverticulum rise directly to the corresponding ascending visceral rami, and do not run back to the junction of gut and diverticulum; this implies that these cells have emigrated after the diverticulum has grown out, i.e. after the 5–7-mm stage; since there are no cell-bodies in the ascending visceral rami after the early stages, it appears likely that they grow out from the denticulate ligament as the diverticulum itself enlarges.

It is suggested above that the nerve-cells of the atrial plexus arise by emigration from the spinal cord, in an analogous manner to the enteric cells of the vertebrates. It is inferred that this process takes place in two stages. First, the cells migrate so far as the denticulate ligament, and then, during the course of post-metamorphic development, these cells pass out yet farther from the central nervous system and come to lie within the gut plexus.

The most striking piece of evidence for this suggestion is the presence of nerve-cell bodies along the course of the ascending visceral rami. It is necessary to consider the question that these may be artifacts, for very similar appearances were long ago observed by Dogiel and Kutchin, near the origin of the dorsal root-nerves in adult animals. These authors used methylene blue techniques, with which these appearances could only be observed after prolonged immersion in the stain; Kutchin concluded that they were certainly post-mortem artifacts. This conclusion is no doubt correct, but since the present material was well fixed (Bouin), and since the cells were only observed in a specific position in animals of a definite size, it is concluded that in this case they are not artifact, and actually represent peripheral nerve-cell bodies. None was ever observed in animals of any age in the position in which they were described by Dogiel and Kutchin.

The function of the system

It is unfortunately correct to say that nothing is known of the function of the complex atrial system, a part of which has been described in this paper. There have been no experiments which provide direct information upon this question. The size of the animal makes it a difficult object for neurophysiological investigation, and also it has not been realized that the system is organized anatomically in a way unique among the chordates.

Since the cells send their axons to the central nervous system, it may be presumed that they are sensory. It was as sensory cells that their discoverer, van Wijhe, regarded them. He wrote to Boeke, '. . . in no case can they be motor, because there are no muscle fibres in the neighbourhood' (Boeke, 1935*a*). Boeke had supposed that they were motor to the muscle-fibres which he stated existed on the gut, but his view of the central connexions of these cells was erroneous.

There are fibrous elements upon the gut, but it is possible that they are connective tissue-cells (Franz, 1927) rather than muscle-fibres. The two cell-types are in amphioxus not easily distinguishable histologically. It is in any case difficult to imagine what muscle-fibres may do on the gut, for the movement of food can be accounted for by ciliary action (Barrington, 1937). One thor (Reeve, 1931) stated that she observed peristaltic movements of the t of *B. belcheri* (the Amoy amphioxus), but this observation has never been nfirmed. I have never seen any movements of the gut which could be erpreted in this way.

However, occasionally a large food-mass collects in the gut, and being tated by the cilia of the ilio-colonic ring, may distort the gut-wall by this vement, giving rise to the spurious appearance of peristaltic movements.

It is not likely, then, that the atrial system on the gut is motor to smooth uscle-cells of the gut-wall. Other motor functions, such as the control of e gut-cilia, or control of secretion, are not only unlikely because of the atomical arrangement of the cells, but also, since no processes appear to oss the basal membrane layer of the gut epithelium, it is not easy to see in hat manner such control might be exerted.

Assuming that the cells are sensory, to what modality they may respond is no means clear. The sensory nerve-cells on the guts of other invertebrates or instance cephalopods, Alexandrowicz, 1928; or Crustacea, Alexan-
owicz, 1909; Orlov, 1924) send processes to the lumen of the gut. It is asonable to regard them as chemoreceptors. But in amphioxus, no processes e seen to cross the basal layer of the gut-cells.

The conclusion that the part of the atrial system on the gut-wall is con-
rned with reception of some stimulus not specifically connected with the t itself (for example, sampling of the water in the atrium), and that e whole system (including the nerve-cells found on the parietal wall of the rium, on the gill bars, and on the endostyle) is a functional unit, concerned th some stimulus of this sort, is not a feasible one. There are more nerve-
lls on the gut than in the whole of the rest of the atrial system. The system the gut, whilst part of the atrial system as a whole, is an obviously specialized rt, no doubt performing some specific function in relation to the gut, hough what this may be is as yet unknown.

There is great need for experiment upon systems of nerve-cells of this sort, hich are organized anatomically in a very different way from those of other ordates. Physiological studies of such systems will not assist the interpreta-
on of the functions of the vertebrate visceral systems, but comparison of the o will prove of much interest when the general questions of the organization nervous units and the origin in evolution of these systems are considered.

oncluding remarks

The condition of the enteric system of nerve-cells in amphioxus (of that ecialized part of the atrial system overlying the gut) is unlike that found in y craniate. Little is known of the connexions of the nerve-cells found upon e gut in Agnatha. It appears from the work of Johnels (1956) that the gut urones are in connexion with vagal and sacral outflows; visceral fibres nerge from both dorsal and ventral roots. It is not known in what way these res connect with the cells of the plexus. The cells themselves (as figured by kuseff, 1897) are rather similar to those of the amphioxus atrial system.

They are multipolar, their processes branch dichotomously, and there are apparent anastamoses between these processes. According to Sakuseff, the axon is not distinguishable from the other cell processes. His figures do no indicate that extrinsic fibres synapse with the cell-body; rather they sugges that the cells give off axons which pass out of the plexus on the gut. The possibility that the system may be arranged in the same way as the amphioxu system cannot be completely excluded.

Little is known of the condition of the enteric systems in the groups tha are perhaps most closely related to the Acrania, the Hemichorda and Tunicata Fedele (1923) claimed that he had observed nerve-cells on the viscera o various tunicates; Millar (1953) did not confirm Fedele's statements in *Cion* (he used iron haematoxylin staining; in my own silver preparations of *Ciona* my observations entirely agree with those made by Millar). In *Salpa* I have observed what may most probably be regarded as visceral nerve-cells; they are multipolar, but their connexions are unknown. It is possible that the amphioxus condition may be found in these groups, but they afford no information on its origin.

Upon the origin of the system, certain points may be made. The first concerns the way in which the system arises in ontogeny. The evidence suggesting that the atrial nerve-cells arise by late emigration from the central nervou system leads to the conclusion that the formation of the atrium in phylogeny has taken place at a later stage than the reduction of nervous potential at the periphery, which must (on any view of chordate phylogeny) have taken place in the chordate line.

From the presumed primitive condition of a diffuse sub-epidermal plexu (as seen in the Hemichorda), the ectodermal material potentially capable o forming nervous material is restricted to a relatively small area of the ectoderm, the neural plate.

This reduction in area of the presumptive nervous material results in late migrations of nerves and cell-bodies to sites which no longer produce nervou material. The atrium forms in amphioxus as an inrolling of the ectoderm resulting in the enclosure of part of the 'external world', and the lining of the organs in the atrium by ectodermal atrial epithelium. Since the nervou material in the atrium is formed by emigration from the central nervou system, it seems that the reduction in nervous potential that has resulted in the need for migration of nervous tissue took place before the atrium originated. It is interesting that Whitear (1957) has remarked that emigration of nervous material probably takes place in *Ciona*. It seems that the atrium formed at a comparable stage in the development of the nervous system in both groups.

Finally, it is almost certain that the atrial nervous system of amphioxus has nothing in common with the enteric systems of nerve-cells found in the vertebrates; there is no possible homology between the amphioxus cells and those found in the plexuses of Auerbach and Meissner. With the change from feeding upon small particles, which are moved through the gut by iary action, to the development of a different feeding method, involving uscular peristaltic movements and their control, it seems that a new system nerve-cells has evolved upon the gut, connected with the central nervous stem in a different manner, and probably of very different function.

It is a pleasure to acknowledge the facilities provided by the Directors and ff of the Stazione Zoologica, Naples, and the Marine Laboratory, Plymouth. is work was carried out during the tenure of the Naples Biological Scholar-p, and of a grant from the D.S.I.R. I am grateful to Dr. William Holmes discussion of several points.

REFERENCES

EXANDROWICZ, J.-S., 1909. Jena. Zeitsch, **45**, 396.
— 1928. Arch. Zool. exp., **67**, 69.
RRINGTON, E. J. W., 1937. Phil. Trans. Roy. Soc. B, **228**, 269.
EKE, J., 1935*a*. Quart. J. micr. Sci., **77**, 623.
— 1935*b*. Z. mikr. Anat. Forsch., **38**, 554.
LE, E. C., 1925. J. comp. Neurol., **38**, 375.
GIEL, A. S., 1902. Anat. Hefte, **21**, 145.
DELE, M., 1923. Atti R. Accad. Lincei, **32**, 98.
ANZ, V., 1927. Ergebn. Anat. Entwick., **27**, 464.
MBLE, H. J., GOLDBY, F., and SMITH, G. M. R., 1957. Nature, **179**, 527.
TSCHEK, B., 1892. Anat. Anz., **7**, 136.
YMANS, J.-F., and VAN DER STRICHT, O., 1898. Mem. Acad. Roy. Belge., **56**, pt. 3.
LL, C., 1927. Phil. Trans. Roy. Soc. B, **215**, 355.
GUE, M. J., 1952. Exp. Cell Res., **3**, 360.
LMES, W., 1947. 'The peripheral nerve biopsy'. In *Recent advances in clinical pathology*, ed. S. C. Dyke. London (Churchill).
— 1953. Quart. J. micr. Sci., **94**, 523.
INELS, A. G., 1956. Acta Zool., **37**, 251.
INSTON, J. B., 1905. Biol. Bull., **9**, 112.
RTISINGHE, P., 1940. Quart. J. micr. Sci., **81**, 521.
NTZ, A., 1909. Anat. Anz., **35**, 387.
TCHIN, H. L., 1913. Proc. Amer. Acad. Sci., **49**, 571.
LLAR, R. H., 1953. '*Ciona*'. L.M.B.C. Mem., **35**, University Press, Liverpool.
LOV, J., 1924. Z. wiss. Zool., **122**, 424.
NTIN, C. F. A., 1952. Proc. Roy. Soc. B, **140**, 147.
EVE, C. D., 1931. Ginling College Magazine, Nanking, January, p. 29.
KUSEFF, S., 1897. Trav. Soc. Nat. St. Petersb., **27**, 29.
N WIJHE, J. W., 1913. Proc. Kon. Akad. v. Wetensch. Amsterdam, **16**, 574.
HITEAR, M., 1957. Ann. Mag. Nat. Hist., **10**, 338.
UNG, J. Z., 1950. *The life of vertebrates*, Oxford (Clarendon Press).

The Effect of reversing the Internal Water-Current on the Spicule Orientation in *Leucosolenia variabilis* and *L. complicata*

By W. CLIFFORD JONES

(*From the Department of Zoology, University College of North Wales, Bangor*)

With two plates (figs. 2 and 3)

SUMMARY

1. Specimens grown in quiet water in an aquarium tank produce the same pattern of spicules as do those occurring naturally. Woodland's hypothesis, that the orientation arises from turbulence in the environment, is thereby disproved.

2. Reversing the internal water-current in excised oscular tubes of *Leucosolenia* has shown that no direct causal relationship exists between the direction of the current and the orientation of the spicules.

3. The spicule arrangement is modifiable by experiment; the orientation is not dependent on the existence of static structural features in the wall of the sponge.

4. Removal of the oscular rim has little effect on the orientation of the spicules subsequently developing in the tube behind.

5. Diverticula develop from bulges, formed largely by a reshaping process. The arrangement of the associated small spicules is essentially the same as for *L. lieberkühnii*.

6. A mechanical hypothesis explaining the orientation of the spicules is briefly described. The formative cell sextet is believed to be oriented by a movement of the mesogloea over the epithelium to which the sextet is contiguous.

INTRODUCTION

A STRONG correlation between the orientation of the spicules in calcareous sponges and the direction of the water-current in the spongocoel was noticed by Haeckel and commented upon by v. Ebner (1887), Minchin (1908), Urban (1906), and Maas (1907): the basal rays of tri- and quadriradiates usually point in the opposite direction to the water-current. Probably the relationship is not a direct one, because at the distal ends of the closed diverticula of *Leucosolenia lieberkühnii* the newly developing spicules can be oriented towards the distal tip before the end has formed an osculum, and consequently before the current has reversed its direction (Minchin, 1908). However, the relationship seemed sufficiently close to justify an experimental investigation, and the following account describes the effect of reversing the internal current in oscular tubes of *L. complicata* (Montagu) and *L. variabilis* (Haeckel) (Minchin, 1904). The method adopted consisted of excising the tubes and mounting them on the tips of fine glass jets through which a current of sea-water was allowed to siphon. This procedure also enabled the effect of removing the oscular rim to be investigated.

A survey of the literature on spicule development has revealed only one

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detailed attempt to explain the orientation of calcareous sponge spicules namely that of Woodland (1905). He believed that the spicule primordia wer initially oriented at random, and that the pattern of the fully grown spicule was to be attributed to the turbulence caused by wave action in the sea-wate around the sponge. This turbulence was assumed to invaginate parts of th sponge wall, or to flex the oscular tubes, thereby causing the developing spicules to rotate into an equilibrium orientation. The hypothesis can b strongly criticized on theoretical grounds (Jones, 1952), but there is no nee here to give a detailed criticism, because Woodland was wrong in assuming that the youngest spicules are haphazardly oriented; in fact the smalles spicules have the same orientation as their fully grown neighbours (Minchin 1908). Moreover, the spicule pattern found when specimens of *L. complicat* or *variabilis* are grown in quiet water in an aquarium tank is, if anything more perfect than that on specimens taken directly from the shore. Woodland's complementary orientating factor, the influence of gravity, also nee not be seriously considered, since the oscular tubes may be found growing a all inclinations to the vertical, without any disturbance of the spicule pattern

MATERIAL AND METHODS

The experiments were carried out with oscular tubes of *L. variabilis* at the Marine Biological Laboratory, Plymouth, from mid-August to mid-September during the four years from 1949 to 1952, and with tubes of *L. complicata* from mid-April to mid-June in 1955 and 1956, at the Marine Biology Station Menai Bridge. The latter species proved the more satisfactory; its larger oscular tubes were less prone to produce diverticula and could be left on the jet for a longer period. The tubes were kept in place by means of a cotton ligature. The wall beneath the ligature tended to disorganize, allowing the tube to slip off the jet. The maximum period of mounting for tubes of *L. complicata* was 3 days, whereas the tubes of the other species could only be retained for a longer period than 46 h if an additional ligature was tied just before the first became ineffective. Longer periods were, however, unnecessary.

Fully expanded oscular tubes were used, since new spicules are not produced by contracted tubes, and in contracting material they may be dislodged from the choanoderm by the crowding together of the fully grown spicules. One cause of contraction is a change in temperature of a few degrees, so that the experiment should be carried out with sea-water at the same temperature as that at which the sponge has been growing. The specimens were therefore left in the aquarium in running sea-water for at least several days after collection in order to adapt them to the temperature of the experiment. The experimental temperature was between 8·5° and 13° C when using *L. complicata*, and 16·5° to 21° C when using *L. variabilis*, taking all the experiments into consideration.

The apparatus employed is shown in the diagram (fig. 1) and requires little explanation. A special clamp made of paxolin is used to support a series of 5 siphon-tubes, each bearing a finely drawn jet. The tubes were made from glass tubing 4 mm thick and of 2·3 mm bore. The jets were about 3 cm long with, at the tip, an internal diameter of 150–350 μ and an external diameter of 250–550 μ. Usually two clamps were used, allowing 10 oscular tubes to be mounted during the same experiment. The two clamps were either set on opposite sides of the same basin, or on the opposite sides of the same reservoir, two receiving basins then being employed. A pressure head of 5–6 cm of water was found to give satisfactory results for both species. Fresh sea-water was used in order to avoid blockage of the oscular tubes by bacterial filaments.

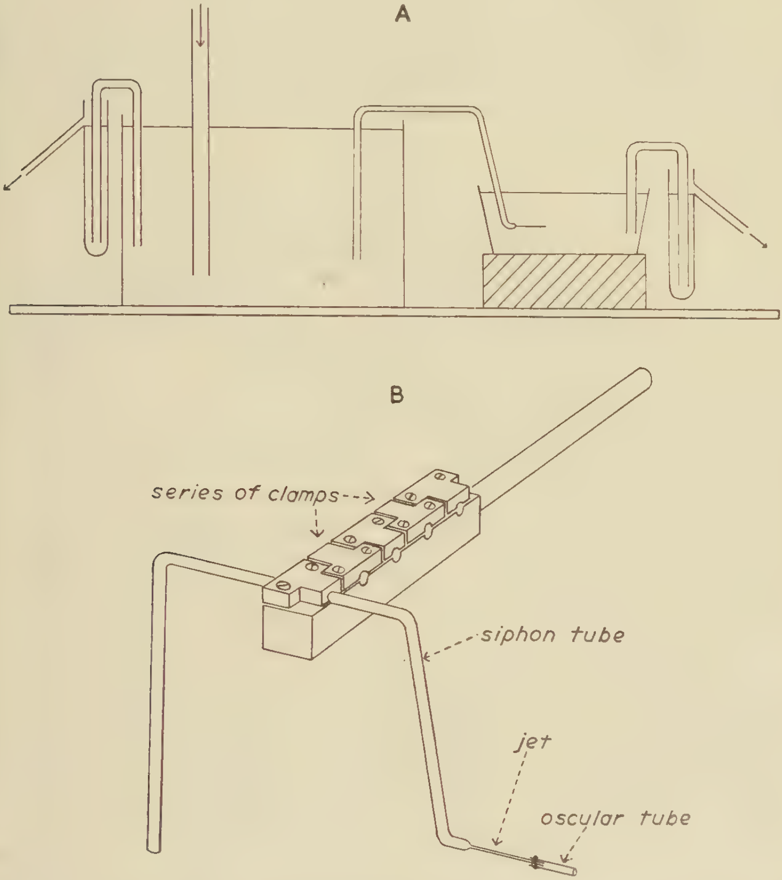

FIG. 1. A, the apparatus used in the jet experiments. Sea-water passes from a reservoir into a basin through a series of siphon-tubes. Each tube has a finely drawn jet at the end, upon which is mounted an oscular tube. Constant levels of sea-water are maintained in the vessels by means of the overflow tubes alongside. B, a siphon-tube in position in one of the clamps.

The method for mounting the oscular tubes was as follows. Some siphon tubes of varying diameter of jet were completely filled with sea-water and aid on a support in a large dish of sea-water, so that the jets were clear of the bottom, but submerged. An expanded oscular tube was excised from the specimen in the dish and the cut end opened by gently squeezing wit forceps. If required, the oscular rim was also removed at this stage, the en being cut at a distance from the edge roughly equal to the diameter of th tube, or at twice this distance. The tube was then pushed over the edge of jet of suitable size and tied on by means of single-stranded thread, prepare by untwining ordinary 6-core cotton.

With a finger over the open end the siphon-tube was next conveyed to th clamp and clamped in position. The oscular tube was taken lengthwis through the air-water interfaces to prevent flexion. At the end of the experiment the siphon-tube was removed from the clamp, again with a finger ove the free end, and the oscular tube was dipped into 90% alcohol. It was take off the jet by sliding the ligature along with forceps.

The tubes may be examined in 90% alcohol, but it was found better t dehydrate, clear, and mount in balsam under a raised coverslip, for then th cells become nearly indistinct and the spicules can be seen clearly. Th spicule pattern is much the same all round the tube, unless diverticula ar present, so that one need only concentrate on the upper surface. The position orientation, and size of the small spicules were plotted on graph paper wit the aid of a squared eyepiece-micrometer, by the use of a 16-mm objectiv and a × 10 eyepiece. Occasionally the use of polarized light, or of a higher-powered objective, was necessary in order to determine the orientation of spicule.

Accurate measurements of the size of the tubes could not be made during these experiments, but sketches were drawn of the shape of many of the tube and notes were made concerning their transparency, which gives a roug guide to their state of expansion.

In all, 34 tubes of *L. variabilis*, 23 of *L. complicata*, and 2 of *L. botryoide* have been accurately mapped, while many more were mounted and gave similar results, although some did not produce crops of small spicules.

There is little point in reversing the direction of the water-current by adjusting the levels so that water flows into the unattached end of the oscula tube, for experiment has shown that the tube simply contracts under such conditions, probably as a result of the decrease in the internal pressure. No small spicules, or few of them, can then be discerned amongst the crowded fully grown spicules.

RESULTS

In the account which follows the tubes which were mounted at their oscular ends will be referred to as 'reversed' tubes, whereas those mounted

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FIG. 2 (plate). A, the distal end of a reversed tube of *L. complicata*, showing the crowded spicules at the edge. On the right close to the edge, is a medium-size T-spicule. The end has partially healed over, the opening now being restricted to the left side. At the middle of the edge can be seen the healing membrane which has drawn the cut edges together on the right side. B, the distal part of the same tube at a different level of focus. Note the many small reversed spicules. The tube was mounted for 47¾ hours. The line measures 100 μ.

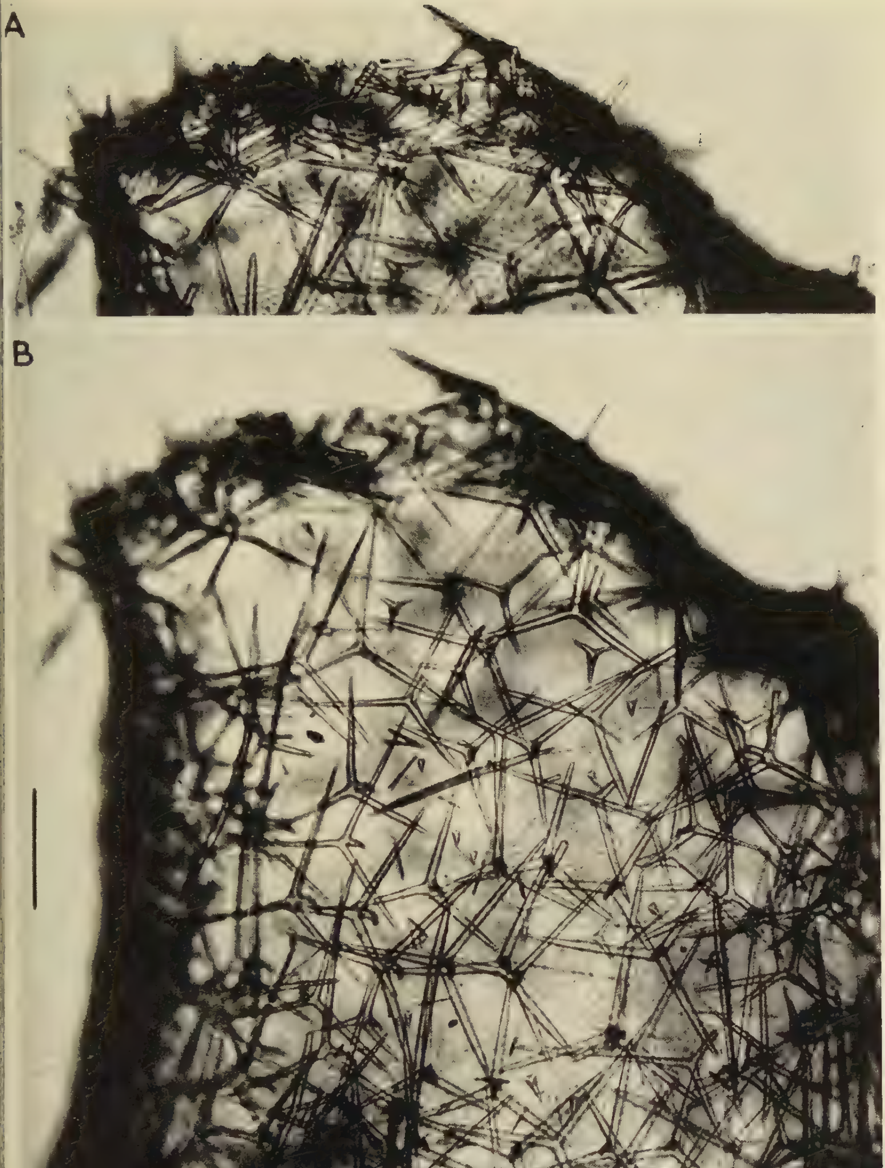

FIG. 2

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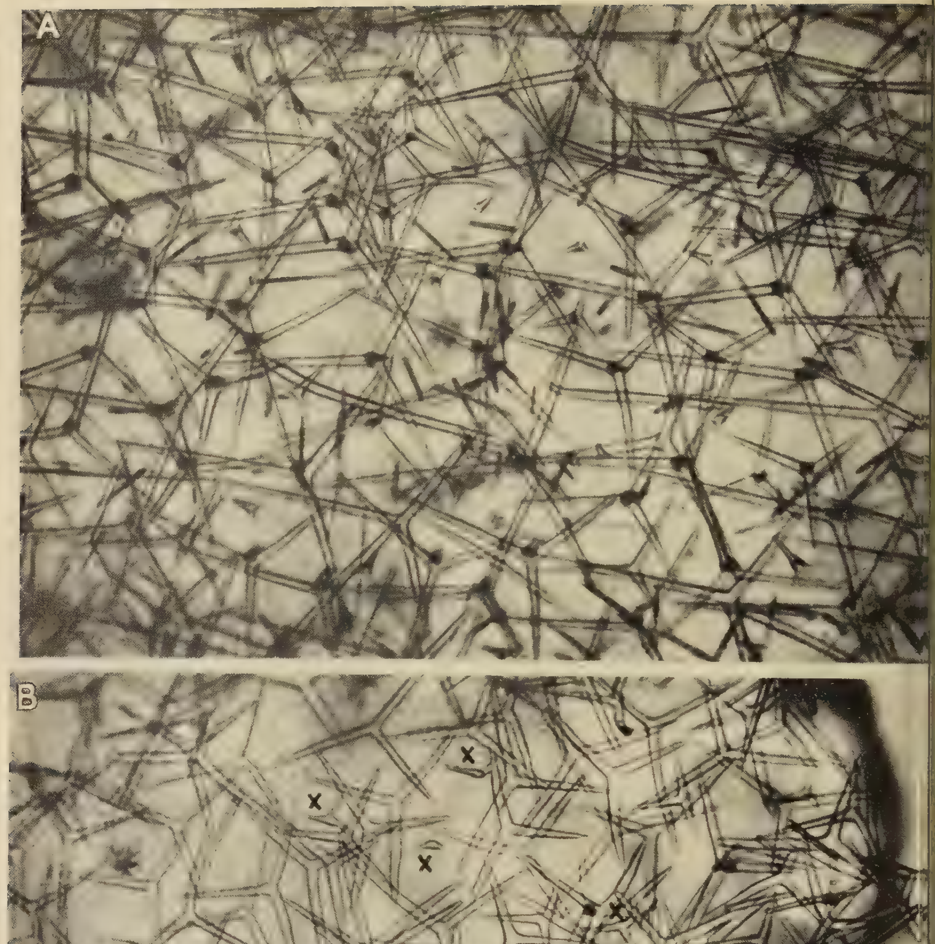

Fig. 3

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y their basal ends will be termed ‘control’ tubes. Of the 34 tubes of *L. ariabilis* that were accurately mapped, 22 were ‘reversed’ and 12 were ‘con- ol’ tubes, while 5 of the former and 9 of the latter had had their oscular rim emoved before mounting. Of the 23 tubes of *L. complicata*, 14 were ‘reversed’ nd 9 were ‘control’, of which respectively 2 and 4 were without their original scular rim.

Both types of tube undergo changes in shape while they are mounted, hough the changes may be small for the control tubes. Invariably the tubes onstrict near the ligature, so as to embrace the jet more tightly, while the eversed tubes also tend to constrict at their distal end. Normally the tubes aper from near the base up to the oscular rim, so that when they are mounted n reverse the end with the smaller diameter will contain water at the higher ressure. Adaptation will therefore result in an expansion near the middle nd a constriction at the free end of the tube. This end may even close up ith the spread of a healing membrane (Jones, 1957) if the internal pressure is nadequate. The distal ends of the control tubes, however, remain open, but ay constrict or expand while the tube is adapting itself to the new conditions. n one case the oscular rim constricted just behind the edge at 11 h after ounting, then slowly recovered and expanded widely during the period rom 52 to 72 h.

The control tubes without their original oscular rim behave much like those ith, except that there is a tendency for the spicules at the cut edge and just ehind it to become crowded together. This tendency is much more noticeable n the reversed tubes, in which quite a thick band of closely packed, partly roken spicules may be found at the distal edge (fig. 2, A). This band can be xplained by the occurrence of longitudinal tension in the surface epithelia oupled with a removal of mesogloea from in between the spicules near the dge, for the act of cutting squashes the surface epithelia together and healing ver the edge is probably a rapid affair. If the cut edge is uneven, one will get ocal patches of crowded spicules as the edge becomes more uniform.

More extreme changes of shape may become apparent, particularly when ubes of *L. variabilis* are used. Quite often they constrict in some regions, or ulge in others, and the bulges may be transformed into diverticula.

he pattern of small spicules arising in the wall of reversed tubes

Reversed tubes which were mounted for more than 17 h (*L. variabilis*) or 5 h (*L. complicata*) were found to have small spicules in the distal part of the ube which were oriented towards the distal edge; that is, their basal rays ointed towards the attached end (fig. 2, B). Fig. 4, A is an example from

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FIG. 3 (plate). A, a group of irregularly oriented small spicules in part of the wall in between he oppositely directed orientation zones of a reversed tube of *L. complicata*, mounted for 7¾ hours. B, the distal end of a reversed tube of *L. variabilis* which was mounted for 80 h. ost of the small reversed spicules present are aberrant in form (indicated by X), some being eficient in a ray and others asymmetrical owing to a rotation of the spicule primordium bout the axis of the basal ray. The line measures 100 μ.

L. complicata. At the distal end there is a zone of reversed orientation of sma and medium-sized spicules extending for nearly one-third of the length o the tube; then a zone in which almost all the small spicules are reverse whereas the larger ones are directed roughly towards the proximal end of th tube; then a zone in which the arrangement of the smallest spicules is confused; and finally a zone extending for nearly half the length of the tube, i

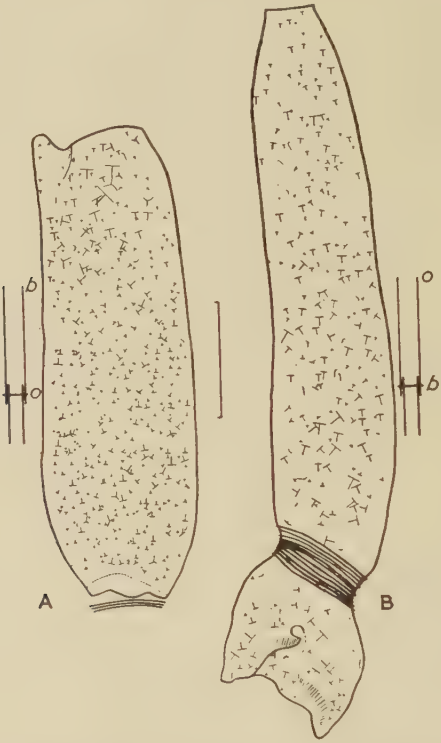
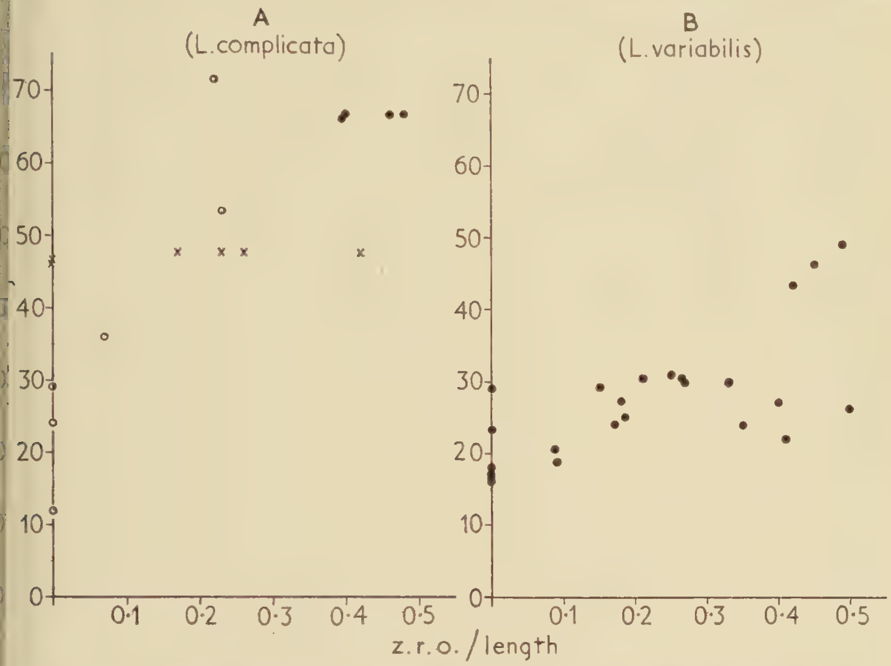


FIG. 4. The pattern of small spicules produced in two tubes of *L. complicata*. For convenienc the spicules (except for obvious aberrants) in this and subsequent diagrams are drawn a T-shaped, the stem indicating the length and orientation of the basal ray. No attempt is mad to give an accurate representation of their form. A, a reversed tube mounted for 47¾ h (description in the text). The broken line indicates the limit of the choanoderm. B, a control tub mounted for 53½ hours. The shading indicates areas of crowded spicules. *o*, osculum; *b*, basa end. The line measures 1 mm.

which nearly all the spicules point towards the original oscular end. The are of 'random' orientation separating the reversed zone and the proximal unreversed zone of another reversed tube is shown in fig. 3, A.

The tube depicted in fig. 4, A was mounted for 47¾ h and comparison wit other reversed tubes of the same species suggests that with longer periods th reversed zones tend to become more extensive. In fig. 5, A the fractio z.r.o./length is plotted against the period mounted for all the reversed tube in which small spicules were found at the distal end. There is considerabl riation, due partly to the arbitrary nature of the measurement, but the versed zones seem to have been established after roughly 30 h. In no case d the reversed zone exceed half the length of the tube.

A tendency for the reversed zone to spread from the edge was also occa- onally shown by the difference in extent of the zones for spicules of different zes. In 3 tubes the zone for the smallest spicules extended farther from the



G. 5. Graphs showing the relation between the hours mounted and the extent of the zone reversed orientation divided by the length of the tube (z.r.o./length). A, *L. complicata*: the bes mounted simultaneously in the separate experiments are indicated by the same mark. *L. variabilis*: the points are derived from tubes used in a number of separate experiments. Small spicules were present in all the tubes plotted.

lge than that for the medium-sized spicules (for example, fig. 4, A), indi- ting a spread of the 'reversed orientation field'. In one tube, however, which as mounted for 72 h, the reversed zone for the smallest spicules was slightly ss extensive than for the larger spicules.

With tubes of *L. variabilis* the results (fig. 5, B) were also variable, but gain there may have been a spread of the reversed orientation field from the ee edge, especially as in 6 tubes the reversed zones had their largest spicules irly close to the edge and the smallest extending the zone to its farthest istance. In other tubes the zone would appear to have either spread rapidly, been established simultaneously all over the distal part of the tube.

No appreciable difference was detected between the reversed tubes with d those without their original oscular rim. In both types the original ientation was maintained in the proximal portion of the tube.

In fig. 6 the length of the basal ray of the largest reversed spicule in a zon of reversed orientation is plotted against the period for which the tube wa mounted (*L. variabilis*). The length tends to increase with longer periods, s that there can be little doubt that the spicules were growing during th experimental period. Again there is much variation, owing to a combinatio of several factors, such as variation in the rate of growth of the spicule

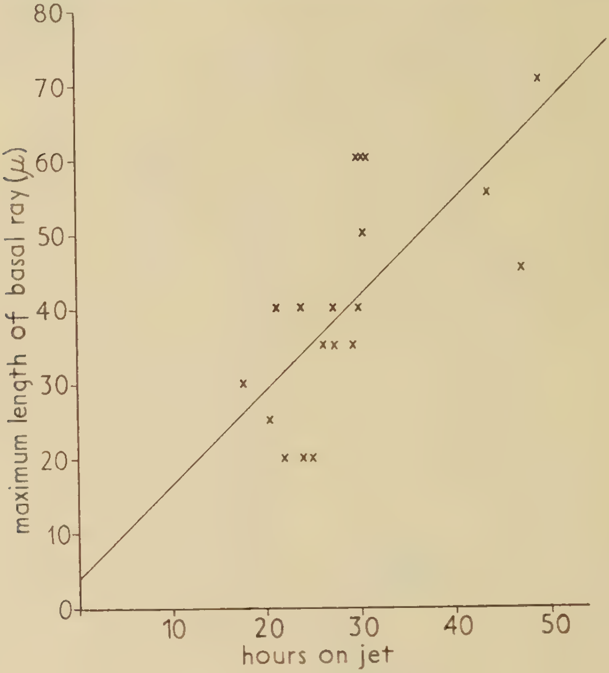


FIG. 6. Graph showing the relation between the hours mounted and the maximum length reached by the basal rays of reversed spicules in the distal part of the reversed tubes of *L. variabilis*. The regression line (correlation coefficient 0·703) cuts the ordinate at 4 μ and has a slope indicating an average growth rate of 1·2 μ per hour.

differences in the relative time of establishment of the reversed orientatio field, and a possible limitation in spicule size due to a delay in the onset o production of new spicules. For these reasons the graph in fig. 6 is of litt use for calculating the rate of growth of the basal ray accurately, and th results for *L. complicata* were even less satisfactory, partly because less poin could be plotted and because some of the reversed spicules had reached th fully grown condition at an indeterminable time before the tube was fixe However, a regression line (correlation coefficient 0·703) has been calculate for the points in fig. 6 and the slope of this line gives the average growth rat as 1·2 μ per h, which is in surprisingly good agreement with the figures o 1·0–1·8 μ per h obtained by photographically recording spicule growth i *L. variabilis* (Jones, 1952; to be published).

The regression line cuts the ordinate at 4μ, from which one would infer at some of the spicules which began growing before the tube was mounted ve suffered reorientation. This is a possibility that must not be neglected, ough it would seem improbable except in the case of very small spicule imordia. Certainly the fully grown spicules do not suffer rotation, and the aph indicates that medium-sized spicules (basal ray about 40μ or more) ere not rotated after periods of up to 20 h on the jet. It is likely that the esogloea is only fluid in the layer close to the choanoderm (Jones, 1956), d that once the spicules have reached a certain size, the thickness of the ys brings the spicule into contact with the outer, firmer layer so that rotation hindered. The form of the larger spicules (a low tripod) also would entail considerable resistance to rotation over the curved surface of the choanoderm.

Another possibility is that some reversed spicules were already present at e time of mounting. Even in healthy oscular tubes the orientation pattern ay not be perfect, particularly at the base, where diverticula may be about form. However, the possibility of spicule reorientation would make the lculation of the time of establishment of the reversed field uncertain, even one knew accurately the rate of spicule growth.

For reversed zones of spicules of different sizes to be readily distinguishole in the same tube, the spread of the reversed orientation field would have be slow. Assuming a constant rate of growth of $1\frac{1}{4}\mu$ per h during evelopment, the basal ray would require 32 h to increase in length by 40μ, uring which time the field would be required to spread from the boundary one zone to that of the other, in practice a distance of less than half the ngth of the tube. As stated above, only 6 tubes out of 22 of *L. variabilis*, d only 3 out of 14 of *L. complicata*, showed this effect.

The concentration of small spicules varies considerably. In some the small icules are abundantly distributed in all regions, whereas in others they may e localized, usually in places where one would expect there to be active rowth. Thus the oscular regions and diverticula are good sources of small icules, while sometimes the distal end has a greater concentration than the iddle of the tube. Some tubes, however, seem to be perfectly expanded and et do not possess small spicules at all. Thus the production of spicules is not ecessarily a continuous process at any particular level of the tube. With . *complicata* a tube with an abundant crop may average 100 small spicules f various sizes per square mm, with the concentration as high as 3 per $10^4\mu^2$ some places. With *L. variabilis* one tube had a crop averaging 190 small icules per square millimetre at the oscular end and 160 per square millietre at the distal end, with local patches containing as many as 4 small icules of various sizes per $10^4\mu^2$. Contracted regions of the wall are poor reas for small spicules.

Aberrant spicules are commonly found in the reversed zones, and fig. 3, B ows a tube in which there is a large proportion of aberrants. There is no eed to explain their form in detail here, but in general it is the result of the ptic axis not lying in an axial plane of the tube (Jones, 1955). This could arise from a rotation of the spicule primordium about the axis of the bas ray, the sextet presumably being tilted laterally, or dislodged, by a laterall directed shear of the mesogloea. In fact many aberrants are probably th result of a clumsy reorientation of small spicules. Some have a ray missin either because a calcoblast has become separated from the sextet, or becaus its secretory activity has been inhibited through the cell being squashed again the epithelium as a result of the abnormal setting of the primordium.

Tubes of *L. complicata* which were left unmounted on the bottom of th basin for 2 days constricted and closed up at their basal end without th

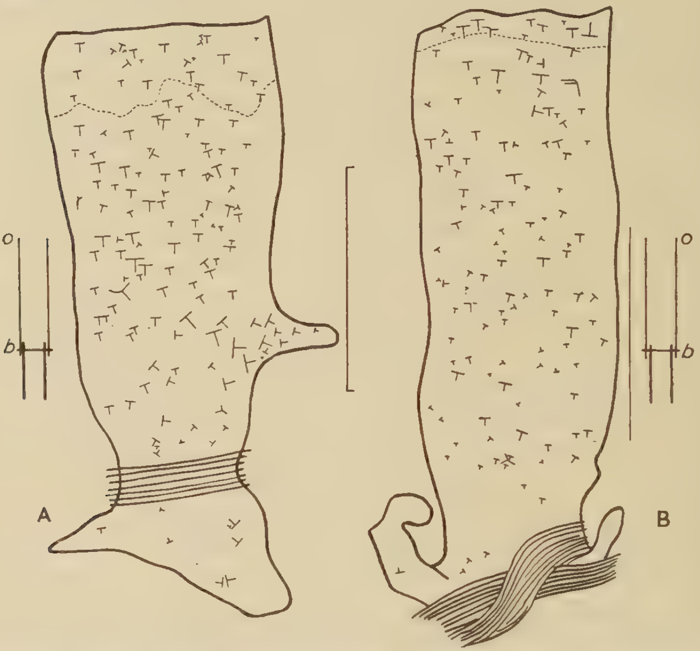


Fig. 7. Patterns of small spicules produced in two control tubes of *L. variabilis*. A, with th original oscular rim. B, without the original oscular rim. Note the reversed spicule at th edge on the right side in B (explanation in the text). The line measures 1 mm.

formation of a reversed orientation zone (fig. 4, B). From the behaviour o similar tubes it is known that a healing membrane (Jones, 1957) forms at th basal edge towards the end of the first day, and this rapidly spreads across th gap, drawing the cut edge inwards. In the mounted tubes the establishmen of a reversed zone at the distal end thus probably depends upon the en remaining open. Presumably the spread of a healing membrane is inhibite by the vigorous internal water-current.

The pattern of small spicules in the control tubes

In the control tubes of *L. variabilis* the spicules were mainly directe towards the oscular end, with perhaps some of the small spicules directe obliquely or laterally, even at sites near the edge. An example is shown i

7, A. The tube was mounted for only 17¼ h and the basal rays of the
erally directed spicules are 25 μ or less in length. Thus they probably com-
nced development at about the time the tube was mounted or later. The
ttern is not much different from that shown in fig. 7, B, a tube from which
original oscular rim was removed just after mounting. One or two of the
all spicules are laterally directed, or reversed, but by far the majority
intain the orientation towards the oscular end of the tube. Again there can
little doubt that the smallest spicules began development during the
perimental period (the tube was mounted for 23¼ h), so that the removal
the oscular rim has interfered but little with the orientation field.

Near the distal edge of the tube in fig. 7, B is a reversed spicule with a basal
y of length 45 μ. Assuming a constant rate of growth of about 1¼ per hour,
is spicule would have been about 16 μ long at the time of mounting, and it
probable that this spicule was reversed by the act of cutting, which must
troduce a considerable amount of mechanical distortion near the edge. Two
her control tubes similarly had one reversed spicule each close to the cut
ge, whose sizes at the time of excision were calculated to be respectively
μ and 28 μ.

At the proximal ends of the control tubes one often finds a confused pattern
small spicules, and sometimes, when the tube has been mounted for a con-
derable time, a zone of reversed orientation. With *L. variabilis* 3 tubes
ounted for respectively 46, 46⅔, and 91 h had a reversed zone at the proximal
ds, whereas 15 tubes mounted for 16¾ to 40 h either had no reversed spicules,
had their small spicules arranged more or less at random in the proximal
gion. With *L. complicata* a reversed zone was present on 4 tubes mounted
r respectively 53¼, 53⅓, 67, and 72 h (fig. 4, B), but not on 4 tubes mounted
r 46, 48, 53¼, and 72 h. The latter tubes displayed a confused pattern at the
tached end, whereas a tube mounted for 43½ h displayed no reversed spicules
all. Thus the establishment of a reversed zone occurs much later in the
ntrol tubes than in the reversed tubes. In the former the cut edge is some
stance behind the ligature, while a free edge only appears at the ligature
y the disorganization of the tissue beneath) some considerable time after
ounting.

All 7 control tubes of *L. complicata* (3 with their original oscular rim) that
ere mounted for more than 53 h had an extensive confused zone. In fig. 4, B
extends for 2·79 mm between the distal zone of 1·2 mm and the proximal
versed zone of 0·66 mm length. In another tube the distal zone was 0·93 mm
ng and the confused zone continued along to the ligature, a distance of
18 mm. It would appear that in such regions of irregular orientation there
d been no overriding orientation field.

The part of the tube behind the ligature is of little value for mapping the
ientation of small spicules owing to its contracted condition and the occa-
nal presence of diverticula. Also some tubes broke free from the ligature
st before fixation and the part behind was not retained. However, in most
the cases mapped the small spicules were mainly directed towards the cut

edge, except in the vicinity of the ligature. The results seem to be consiste with those obtained at the distal ends of the reversed tubes, as would perhap be expected since the presence of the glass jet prevents the closure of th cut end.

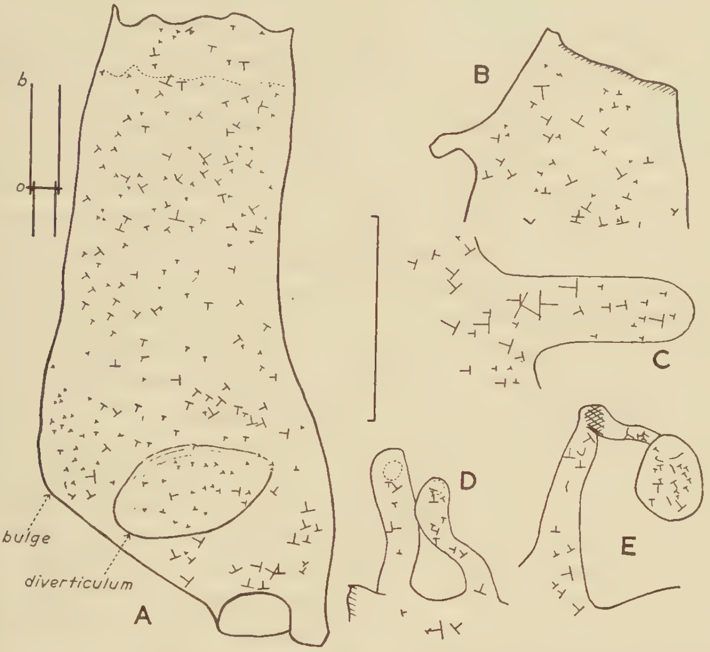
One control tube of *L. complicata* was mounted on a jet which was fille with air, so that no current could siphon through. After 46 h this tube w no longer cylindrical, but partially constricted at two levels besides th attached end. The resulting pattern of small spicules was irregular over mo of the tube, with some reversed spicules here and there. The arrangement the basal end was also confused. This tube indicates the importance of maintaining an adequate pressure inside the spongocoel.

The pattern of small spicules in bulges and diverticula

Diverticula were quite often produced on the experimental tubes and th pattern of their fully grown spicules reveals their mode of origin. The spicul on the surface facing the oscular rim are directed towards the base of th diverticulum, whereas those on the surface facing the base of the main tub are directed towards the apex. On the two sides the spicules are oriente across the diverticulum, in the direction roughly towards the osculum. Th diverticulum has thus arisen from part of the wall of the main tube which ha bulged outwards and then constricted at the perimeter of the bulge. Th bulging is probably the result of a localized contraction of the internal ep thelium, which has been shown to have contractile properties (Jones, 1957 However, amongst the fully grown spicules may be seen small ones whic point away from the apex of the diverticulum regardless of their position i its wall (fig. 7, A). Thus the orientation pattern for the small spicules is th same as for the closed diverticula of *L. lieberkühnii* (Bidder, 1891; Minchin 1908); and more so, because the small spicules may develop in the opposit orientation both before (at least in *L. complicata*) and after an osculum ha appeared at the distal end (figs. 8, C, D).

The tendency for the small spicules to be directed away from the ape may also be seen in the radiating pattern on bulges of the wall (fig. 8, A though the orientation is not always regular. Even the parts around the diverti culum may have small spicules directed away from it (figs. 8, B, 7, A). I fig. 8, A is shown a tube which had begun to produce diverticula before th start of the experiment. One diverticulum (depicted in the figure) is close t the proximal end, whereas two others are much nearer the distal end an sited, one on each side, on the opposite surface. Zones of reversed orientatio extend from the distal edge up to each diverticulum, the one shown coverin more than half the length of the tube. This was the only case in which reversed zone reached beyond the half-way level, and, as indicated above, i did so on part of the surface only and its presence is to be correlated with th formation of the diverticulum. Many of the reversed spicules in this zon must have been present at the time the tube was mounted, judging from thei size, for reversed spicules of 40 μ length occur, even though the tube wa ounted for only 21 h. This experiment indicates the importance of mounting bes without diverticula when studying the establishment of a reversed ne at the distal end of the tube.

Occasionally the diverticula rounded off completely to form spheroidal jects at the tips of fine tubes (fig. 8, E). There can be little doubt that such bjects, broken away from the parent tube, would be capable of distributing ie species. Vasseur (1879) has described this process of asexual reproduction *L. botryoides.*



IG. 8. A, reversed tube of *L. variabilis*, mounted for 21 h. Note the radiating pattern of small icules in the bulge and diverticulum. B, distal end of a reversed tube of *L. variabilis*, ounted for only 17¾ h. A zone of reversed orientation has not been established at the distal lge. Small spicules radiate from the base of the diverticulum. C, a closed diverticulum from tube of *L. complicata*; the small spicules are directed towards the tip. D, two diverticula om a tube of *L. variabilis*, each with an osculum at the tip. Some small spicules are oriented wards the oscula. E, spheroid formed at the distal end of a diverticulum (*L. variabilis*). The ne measures 1 mm. Shading indicates areas where fully grown spicules are crowded together. The broken line indicates the edge of the choanoderm. *b*, basal end; *o*, osculum.

ormation of a new oscular rim at the cut end

As stated above, there was a tendency for the spicules to become crowded ogether at the cut edge. The boundary of the choanoderm, moreover, ended to separate from the edge, leaving a clear zone. This occurred in eversed tubes as well as in control tubes without their original oscular rim. he choanocyte boundary was often very uneven, but with *L. variabilis* in ost cases an obvious clear zone seems to have been established by about

20 h. This zone is to be regarded as a new oscular rim, and the occurrence o developing T-shaped spicules near the cut edge confirms that the growt processes normally associated with an oscular rim had been in operation.

With tubes of *L. complicata* the tendency for a clear zone to form at the cu end was much less apparent. Even after 72 h on the jet a control tube still ha no clear zone and the same was true for 2 other tubes mounted for abou 53 h. Only in 3 reversed tubes, mounted for 36, 67, and 67 h, was there foun a narrow zone extending for respectively 13–93 μ, 13–66 μ, and 40–55 μ. Sinc a zone of reversed orientation was found in 5 tubes in which the collar-cell reached the free edge, while some T-spicules were present close to the edg on 2 reversed and 2 control tubes which did not have a clear zone, it may b concluded that the presence of the latter is not a prerequisite of the establishment of the growth processes controlling the form and orientation of spicules There is evidence that the collar-cells rest upon a porocyte epithelium (Jones 1957), so that the position of the collar-cell limit need not have significance i respect to the spicule pattern.

When the cut end closes up a clear zone is not to be seen. On the contrar the spread of the healing membrane across the gap would tend to draw th choanocyte-region in the direction of the cut edge.

DISCUSSION

Three conclusions concerning the mechanism of orientation may be derive from the above account.

(1) The reversed orientation of the small spicules at the distal ends of th reversed tubes, and the random orientation in various regions elsewhere prov that the mechanism of orientation is a dynamic one; the orientation at particular site is not dependent upon the existence of static structural feature in the wall, but is controlled by factors which are easily modifiable.

(2) The maintenance of the original orientation field at the proximal en of the reversed tubes, despite the reversal of the internal water-current, show that there is no direct causal relationship between the direction of the curren and the spicule orientation.

(3) Since the removal of the oscular rim does not appreciably disturb th orientation field, it is unlikely that the rim is controlling the spicule orientation. It might have been supposed that the calcoblasts were guided by chemical or bio-electric gradient established by the metabolic activity of th oscular rim. However, this is unlikely because three apparently equal cell (Minchin, 1908) participate in the formation of a triradiate spicule and it i difficult to explain how the three similar cells could dispose themselve appropriately across an uniaxial gradient. Moreover, the spicule orientation i maintained right up to the oscular edge, where there is nothing of histologica distinction to warrant the conclusion that it constituted an active source o bio-electric potential or of a chemical substance.

The production of aberrant spicules has been explained by a laterally o liquely directed shear of the ambient mesogloea, and the normal arrange- ent of a sextet containing a spicule primordium also suggests that it is the sult of a shear of the mesogloea in the direction of the oscular edge (Jones, 54). The latter shear could arise either through a greater expansion of esogloea in the more basal regions of the tube, or through a greater growth the epithelium (contiguous with the sextet) at the oscular end, or through combination of these two factors. The hypothesis will be presented in more tail in a later paper, but for the present it can be stated that the results scribed above can all be explained in similar terms. Thus in reversed tubes e mesogloea can be assumed to spread into regions of extension at each end the tube, while the shear distally would be aided by the process of spicule- owding at the distal edge. The formation of a bulge is probably the result a localized contraction of the internal epithelium, which, coupled with a cretion of mesogloea at the apex of the bulge, would result in a radiating ttern of shear. Subsequently the epithelium at the apex of the diverticulum ould begin active cell-division, the secretion of mesogloea there would cease, d the direction of shear would be reversed. Eventually a new osculum would en at the tip and development would proceed as in the main oscular tube. ith the unmounted tubes the spread of the healing membrane across the t basal end would draw the epithelia towards the base and thus produce a ear which would maintain the orientation towards the oscular end.

No small spicules were produced in which the optic axis was perpendicular the wall of the tube, even in regions of irregular orientation where there ould possibly have been no shear (fig. 3, A). Perhaps the sextets only start secrete their spicules when there is a movement of mesogloea around them, tremely localized movements being the cause of the irregular orientation such regions. Alternatively the stiffness of the outer layer of the mesogloea ay be responsible for the tilting of the sextet when there is no shear, there ing insufficient room between this layer and the internal epithelium for the ter three cells to perch on top of the inner trio. If this be the case, the riation in inclination of the optic axis along the tube (Jones, 1954) may be termined by differences in the thickness of the softer mesogloeal layer. hatever may be the explanation for the inclination of the optic axis, a process shear is a likely cause of the orientation of the sextet.

As stated above, the experiments were first carried out with *L. variabilis* at e Marine Biological Laboratory, Plymouth; and later, with *L. complicata*, the Marine Biology Station, Menai Bridge, Anglesey. During the first three ars I was supported by a grant from the D.S.I.R. and the work was super- sed by Professor Sir James Gray. I wish to record my thanks to my super- sor and to the Heads of the departments concerned.

REFERENCES

DDER, G. P., 1891. Quart. J. micr. Sci., **32**, 625.

BNER, V. VON, 1887. S.B. Akad. Wiss. Wien., **95**, 55.

NES, W. C., 1952. Ph.D. Thesis, Cambridge.

JONES, W. C., 1954. Quart. J. micr. Sci., **95**, 33.
—— 1955. Ibid., **96**, 129.
—— 1956. Ibid., **97**, 269.
—— 1957. Ibid., **98**, 203.
MAAS, O., 1907. S.B. Ges. Morph. Physiol., München, **23**, 82.
MINCHIN, E. A., 1904. Proc. zool. Soc. Lond., **2**, 349.
—— 1908. Quart. J. micr. Sci., **52**, 301.
URBAN, F., 1906. Arch. Naturgesch., **72**, 33.
VASSEUR, G., 1879. Arch. Zool. exp. gén., **8**, 59.
WOODLAND, W., 1905. Quart. J. micr. Sci., **49**, 231.

he Ultra-fine Structure of Lipid Globules in the Neurones of *Helix aspersa*

By J. T. Y. CHOU *and* G. A. MEEK

(*From the Cytological Laboratory, Department of Zoology; and Department of Human Anatomy, University Museum, Oxford*)

With four plates (figs. 1–4)

SUMMARY

The three kinds of lipid globules recognizable in the living neurones of *Helix aspersa* ve been examined under the electron microscope. The globules of the kind that can stained blue with methylene blue during life are seen in electron micrographs as heres or spheroids, with concentric lamination, after calcium-osmium fixation. After ation with sucrose-osmium laminated crescentic bodies are seen instead; these appear be formed by distortion of the 'blue' globules. The yellow globules contain electron- nse material, and sometimes appear reticular. It is possible that the yellow globules y originate by transformation of some of the 'blue' globules. The colourless globules nerally appear as crenated objects; this appearance may be a shrinkage artifact.

Apart from the mitochondria and the three kinds of lipid globules described, no ner object large enough to be identified with the light microscope has been seen in cytoplasm.

INTRODUCTION

T has been shown (Chou, 1957 *a*, *b*) that there are three kinds of lipid globules in the neurones of the snail, *Helix aspersa*, and that they are dis- guishable from each other by the use of vital dyes and by histochemical sts. One kind, called the yellow globules, have naturally a yellow colour ving to the presence of carotenoid. Another kind, called the colourless obules have no natural colour and cannot be coloured by vital dyes. A third nd, called the 'blue' globules, have no natural colour but are readily dyed life by methylene blue, brilliant cresyl blue, or Nile blue.

The purpose of this investigation with the electron microscope was to find idence as to whether any of these globules, and if so which, might be con- rned in the appearance called 'Golgi apparatus' (or 'dictyosomes') in Golgi eparations; or whether there might be some other cytoplasmic inclusion, t visible in life, that was responsible for appearance of the 'Golgi apparatus'.

MATERIAL AND METHODS

For this investigation, the cerebral ganglia of *H. aspersa* were used. After e animal had been killed by decapitation, the cerebral ganglia were removed mediately and placed in fixative. Two fixatives were used:

1. Sucrose-osmium. 1% osmium tetroxide+0·35 M sodium-veronal acetate buffer+0·22 M sucrose (Caulfield, 1957).
2. Calcium-osmium. 1% osmium+0·35 M sodium-veronal acetate buffer +1% calcium chloride.

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The ganglia consist of two groups of neurones. Aggregations of neurone were dissected out in the fixatives and left in them at 0° C for 1 h. The materia was dehydrated in graded alcohols and embedded in *n*-butylmethacrylate the latter was polymerized for 12 h at 45° C.

Thin sections were cut on a Porter–Blum microtome and mounted o carbon-coated grids. In some cases, immediately adjacent 2 μ sections wer cut. These thick sections were transferred with a fine bristle brush to a clea coverslip or slide, and were then dried with warm air.

Electron micrographs of the thin sections were taken with a Siemen 'Elmiskop I' at 40 kV or 60 kV. For direct microscopy, the thick sections wer placed in 70% alcohol for a few minutes and then left for 10 min in a saturate solution of Sudan black B in 70% alcohol. They were differentiated in 70% alcohol for 5 sec and in 50% alcohol for 1 min; they were washed thoroughl in distilled water and finally mounted in Farrant's medium. Two-micro sections were also mounted in unpolymerized methacrylate and examined b phase-contrast.

RESULTS

In electron micrographs one sees mitochondria and three other kinds o cytoplasmic inclusions large enough to be studied by light microscopy Comparative studies of adjacent thin and thick sections under electron an light microscopy were made, and the same cells were readily identified (fig 1, A, B). It was difficult, however, to identify with any certainty the sam individual lipid globules in the thick and thin sections. The three kinds o cytoplasmic inclusions can, however, be identified in electron micrograph by their positions in the cells (see fig. 1 in Chou, 1957*a*).

Colourless globules. One of the kinds of cytoplasmic inclusions is seen i electron micrographs in the axon, arranged in rows resembling beads strun together. These are obviously the colourless globules, which are known fro previous studies with the light microscope to be arranged in this way. The occur also here and there in the cytoplasm of the cell.

The colourless globules are shown in fig. 2, A, B. The micrographs repre sent axons cut transversely (A) and longitudinally (B). The electron-dens objects shown in these micrographs are the colourless globules. They ar about 1 μ in diameter. This size corresponds closely with that obtained b light microscopy. They appear in most electron micrographs as crenate objects. When calcium-osmium is used as fixative, the crenated appearanc is even more pronounced (fig. 4, A). The crenation is almost certainly a artifact. The colourless globules are bounded by a simple membrane. The

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FIG. 1 (plate). A, a low-power electron micrograph of the neurone of *H. aspersa* to show th the crenated colourless globules (*c*) are distributed near the axon (*a*) and the large yellow globul (*y*) are near the axon hillock (*ah*). The nucleus (*n*) is partially shown in this micrograph.

B, a consecutive 2 μ section to show that in the same cell coloured with Sudan black an photographed by light microscopy all the lipid globules (*l*) are spherical and colour with Sud black. Calcium-osmium fixation.

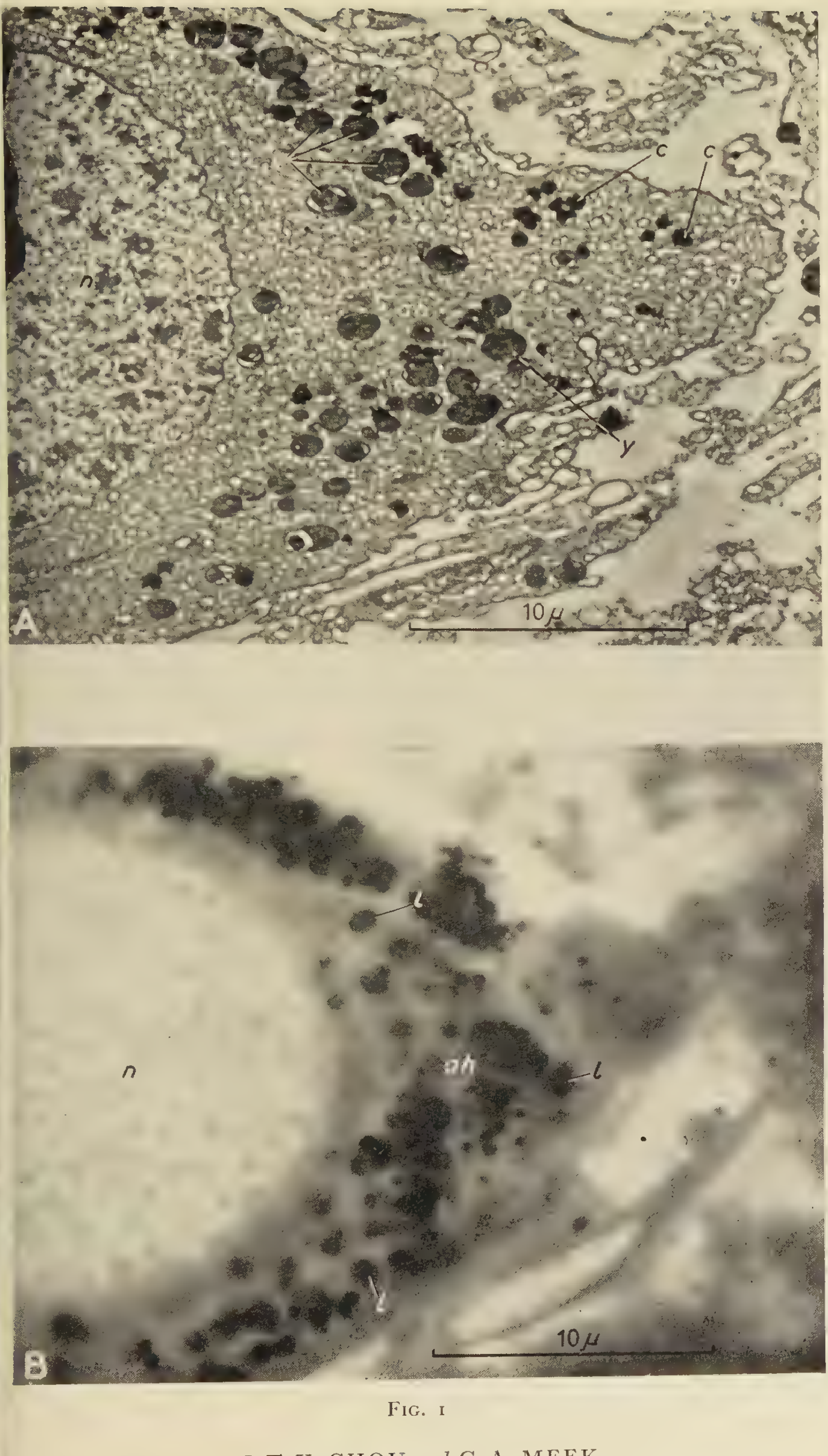

FIG. 1

J. T. Y. CHOU *and* G. A. MEEK

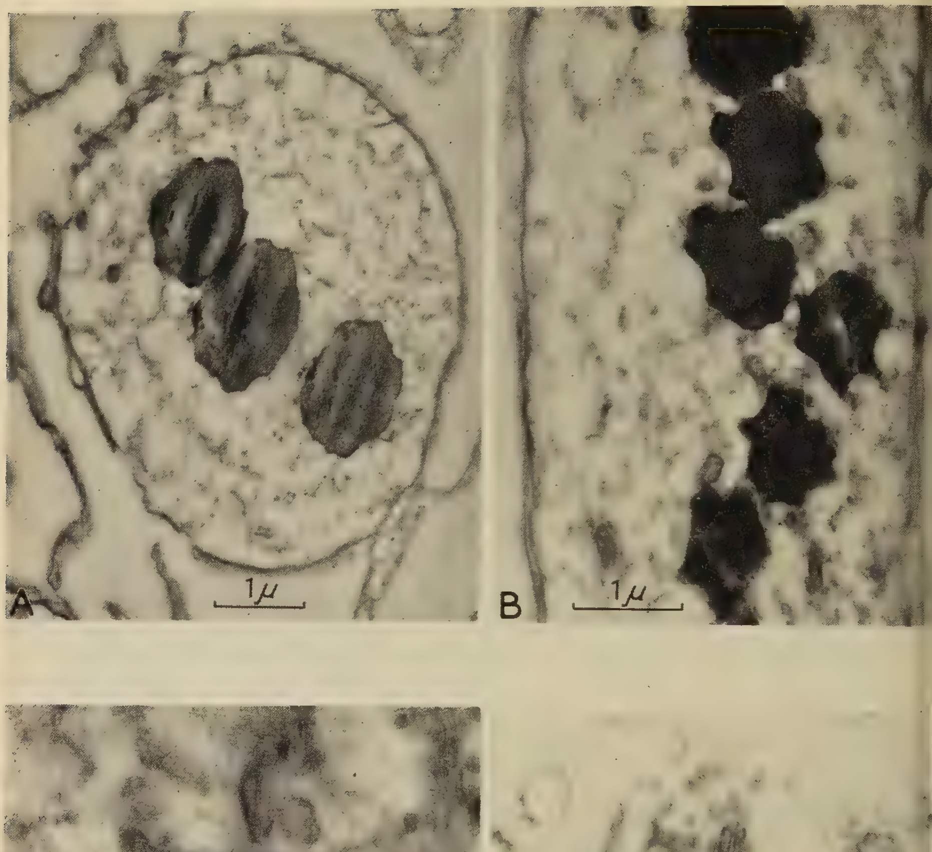

Fig. 2

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ntents appear as a solid mass. The corrugated appearance generally seen the substance of the globules is an artifact of sectioning, as the axis of rrugation is always parallel to the knife-edge.

Yellow globules. The yellow globules can be recognized in electron micro-aphs by three criteria already established by light microscopy. (1) They do t occur in small neurones; (2) they are particularly abundant in the axon llock of large neurones; and (3) they are nearly always larger than the other o kinds of globules (diameter about 1·5 to 2 μ).

The yellow globules are electron-dense, but less so than the colourless obules. They appear to be bounded by lamellae. In preparations fixed with lcium-osmium these lamellae are particularly well preserved and 'stain' tensely with osmium. With this fixation lamellae are also visible in the in-rior of the yellow globules (fig. 3, C, D). The rest of the material of these obules appears to be structureless, or sometimes reticular.

Some of the yellow globules carry one or more 'satellites'. These are also ounded by layers of lamellae, but their internal parts are either vacuolated ig. 3, B) or show little electron-density. These satellites resemble the 'blue' obules.

'*Blue*' *globules.* These are known from studies with the light microscope be evenly distributed throughout the cytoplasm, but they are absent from e axon. They occur also in small neurones. These characters enable them be distinguished in electron micrographs.

The two fixatives used in this investigation give different appearances to e 'blue' globules.

In calcium-osmium preparations the 'blue' globules appear spherical or void (fig. 4, B). They consist of a laminated electron-dense external part, or ortex, and a central part or medulla that shows little electron-density. Each mella appears to consist of two electron-dense layers with a less electron-ense layer in between, each of the three layers being about 20 Å thick. he dense layers appear to consist of irregularly arranged particles of lectron-dense material. The total thickness of each lamella is about 60 Å. The istance between the lamellae varies in different globules from 100 to 200 Å. he thickness of lamellated cortex is often variable in different parts of the ame globule (fig. 4, B).

When sucrose-osmium is used as fixative, the 'blue' globules do not retain heir globular shape, but appear as crescentic objects (fig. 2, C) or even (in

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FIG. 2 (plate). Electron micrographs of lipid globules in neurones of *H. aspersa*. Sucrose-smium fixation.

A, transverse section of axon showing three colourless globules.

B, longitudinal section of axon showing a row of colourless globules.

C, a 'blue' globule showing as an 'opened' ring, consisting of laminated membranes. The ner substance of the globule is not electron-dense.

D, another form of the 'blue' globules showing as a parallel double laminated membrane d associated with vacuoles. This appearance is probably due to a tangential section through e surface of the burst 'blue' globule.

section) as rods (fig. 2, D). The lamellae are similar to those seen in calcium-osmium preparations. Small vesicles are often seen in addition to the lamellae (fig. 2, D).

Thick sections, fixed in calcium-osmium, were coloured with Sudan black: all the lipid globules became homogeneously blue-black (fig. 1, B). No crescentic, curved, or cap-shaped structures appear in these preparations.

Mitochondria can be identified in the electron micrographs (fig. 3, C). They are filamentous and are found in all parts of the cytoplasm; they are better preserved in calcium-osmium preparations than in those fixed in sucrose-osmium. The limiting membrane of the mitochondrion is distinguishable, and also the matrix and cristae.

DISCUSSION

From the present study, it appears that the crescentic laminated objects seen by electron microscopy in the nerve cells of *H. aspersa* result from an artificial modification of the 'blue' globules visible in living cells. In a previous study (Chou, 1957*b*) histochemical tests have demonstrated that these globules contain only phospholipids. From the present study, it has been shown that calcium-osmium fixation can preserve these globules as spheres comparable to those seen in living cells. However, we have seen from this present work that structures consisting of laminated membranes or lamellae tend to be formed usually at the periphery of the globules. These lamellae are probably due to the presence of phospholipids. Ross and Chou (1957) have brought forward strong evidence that the 'blue' globules contain a considerable proportion of water. This may be correlated with the fact that the central part of the 'blue' globule has a low electron-density. It cannot, however, be regarded as certain that the lamellae were situated at the periphery of the globule during life. During the processes of fixation, dehydration, and embedding for electron microscopy, water may be removed and the globules may then burst, thus forming crescentic objects. Calcium has a tendency to preserve the concentric lamellar arrangement of the phospholipids, and thus to cause the globules to retain their spherical or spheroidal shape.

Young (1932, 1953, 1956) has reported that the neutral red globules in the neurones of cephalopods can be swollen or shrunken osmotically, and he

---

FIG. 3 (plate). Electron micrographs showing a series of changes of the 'blue' globules into yellow. Calcium-osmium fixation.

A, shows that certain internal substances (probably carotenoids, protein, or lipids other than phospholipids) are strongly electron-dense. The laminated membranes (*lm*) begin to become entangled in the globule with other substance. A mitochondrion (*m*) with long cristae is shown.

B, similar to A, but the electron-dense material in the globule has increased in amount. A satellite (*s*) is probably attached to this globule.

C, a fully developed yellow globule (*y*) containing very electron-dense material and also laminated structures (*lm*) (probably phospholipids). A mitochondrion is clearly shown.

D, a globule that appears to certain reticular structures and also parallel laminated membranes.

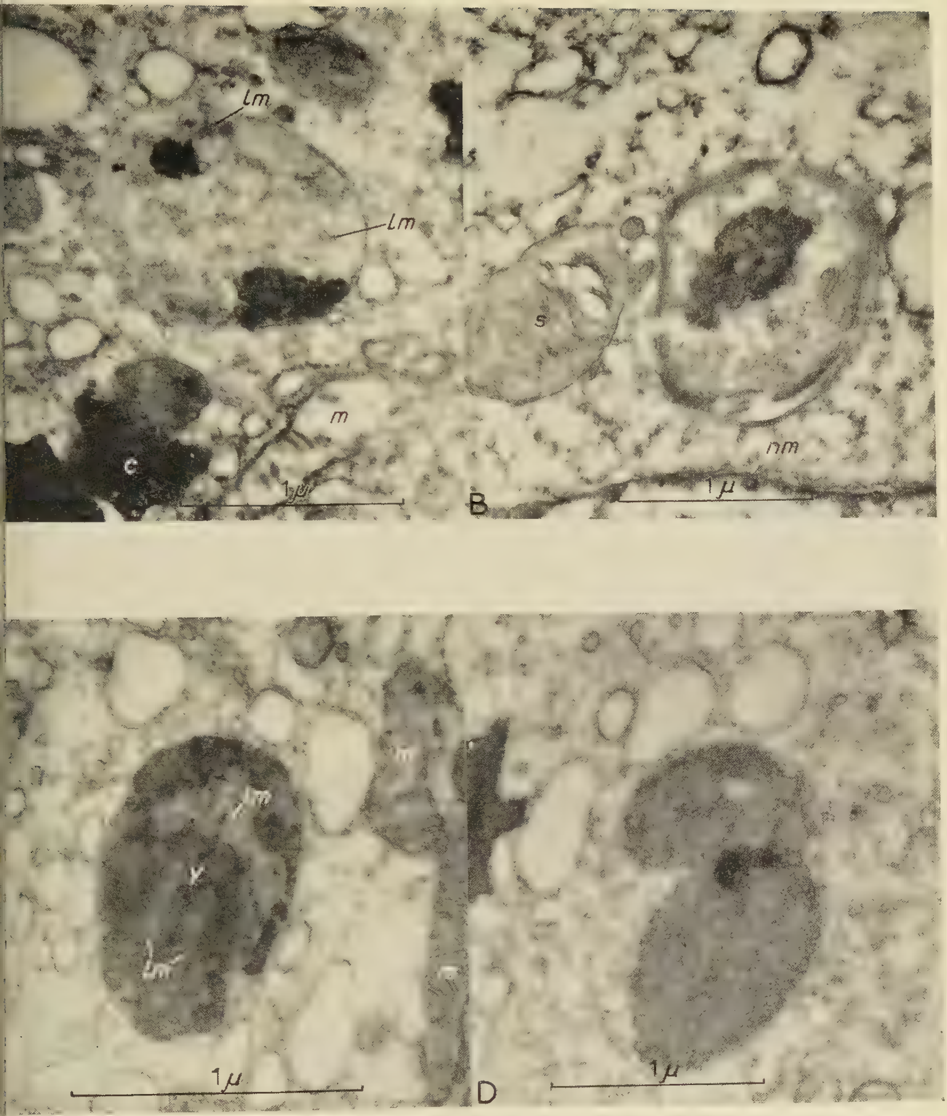

Fig. 3

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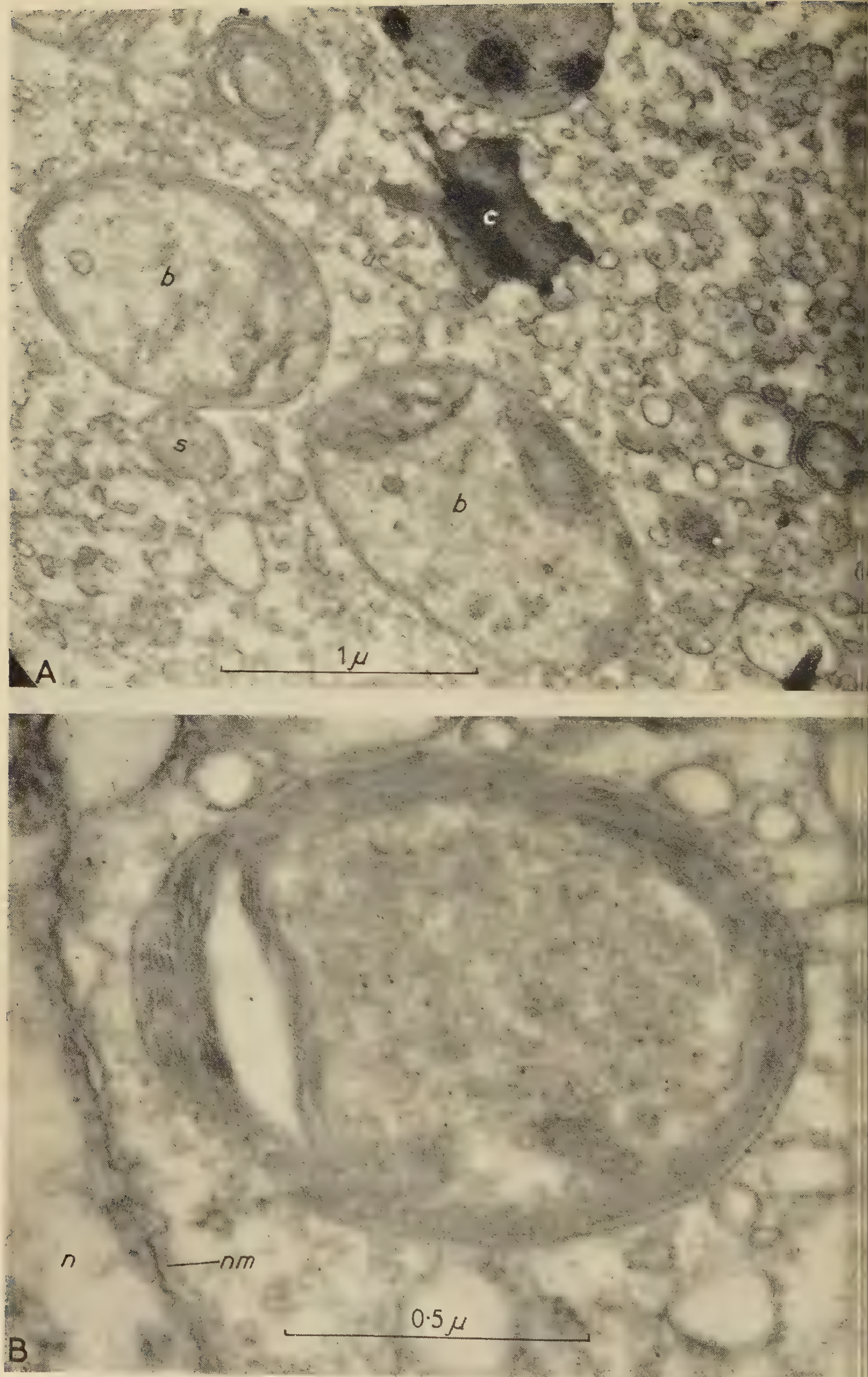

FIG. 4

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ncluded that a semi-permeable membrane might be present. This mem-
ane might be formed of concentric lamellae similar to those that we have
und in the neurones of *Helix*.

From the present electron microscope study, it is also interesting to note
at our findings concerning the 'blue' globules agree with Schmidt's belief
939) that, in the presence of water, certain lipids tend to arrange them-
ves in the form of concentric lamellae (see Baker, 1957).

The thickness of each membrane is consistent with the hypothesis that it
presents a bimolecular phospholipid layer. The 'myelin' globules that exude
om red blood corpuscles ingested by macrophages show a submicroscopic
ucture similar to that of the 'blue' globules (Policard, Bessis, and Breton-
orius, 1957; Stoeckenius, 1957). These authors interpret the membranes
the 'myelin' globules as bimolecular layers.

Hodge (1956) and Hodge and others (1956) described similar lamellar
uctures in chloroplasts of *Zea mays*. These lamellae present an almost
entical granular, double-membrane appearance, and are believed by them
be oriented phospholipid or lipoprotein molecules; but the whole arrange-
ent of the lamellar membrane is not concentric like that of the 'blue' globules
Helix.

The structure of these three kinds of lipid globules strongly suggests that
e yellow globules originate from the 'blue' globules. In calcium-osmium
eparations, the 'blue' globules of the small neurones (like those of the large)
pear as spherical objects consisting of strongly electron-dense, laminated
embranes. These membranes enclose certain substances which, perhaps,
ve a high content of water and which are not 'stained' at all by osmium (fig.
B). During the development or growth of the cells, carbohydrate, protein,
d lipids other than phospholipids, particularly cerebrosides and carotenoids,
ay aggregate in the 'blue' globules. These organic substances, being less
turated, may take up osmium to a greater extent. The latter fact would account
r the greater electron density of the internal matrix of the globules during
evelopment from 'blue' to yellow. Fig. 3, A–C is a series of three electron
icrographs showing the aggregation of electron-dense material in the body
the globules. When the 'blue' globules are completely filled with these
ense substances, the laminated phospholipid membranes are no longer very
rominent. It is possible that they may be dispersed or incorporated into the
ther substances in the globules (fig. 3, C). In many cases the layers of lamin-
ed structures enclosing a certain amount of electron-dense material may
ill be identified. In some globules the dense material appears to have a
ticular structure (fig. 3, D).

FIG. 4 (plate). Electron micrographs of lipid globules in neurones of *H. aspersa*. All these
icrographs were taken from neurones fixed in calcium-osmium preparations.

A, a group of lipid globules. The crenated colourless globule (*c*) appears to be more electron-
nse than the two 'blue' globules (*b*). One of the 'blue' globules seems to bear a small satel-
e (*s*).

B, a 'blue' globule showing its ovoid form as seen by light microscopy. The inner substance
the globule is not electron-dense. *n*, nucleus; *nm*, nuclear membrane.

The colourless globules seem to be quite independent of the other tw types of globules. They are electron-dense, possibly owing to the presenc of unsaturated fatty acids, and appear to be devoid of any laminated membranes.

The duplex character of lipid globules appearing in living cell and in fixe preparations when examined by light microscopy has been summed up b Baker (1957). In our studies of these lipid globules, we have found that perfectly spherical globules can apparently be changed into crescentic forms unde the influence of the particular fixative used.

In 'Golgi' preparations, the black material is presumably osmium or silve deposited in or on the lamellae of the 'blue' globules. The findings with th electron microscope confirm the conclusion reached by one of us (Chou, 1957*a* that the 'Golgi apparatus' ('dictyosomes') of these cells is represented in lif by the 'blue' lipid globules.

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REFERENCES

Baker, J. R., 1957. Nature, **180**, 947.
Caulfield, J. B., 1957. J. Biophy. Biochem. Cytol., **3**, 824.
Chou, J. T. Y., 1957*a*. Quart. J. micr. Sci., **98**, 47.
—— 1957*b*. Ibid., **98**, 59.
Hodge, J., 1956. J. Biophy. Biochem. Cytol. suppl., **2**, 221.
—— McLean, J. D., and Mercer, F. V., 1956. Ibid., **2**, 597.
Policard, A., Bessis, M., and Breton-Gorius, J., 1957. Exp. Cell Res. **13**, 184.
Ross, K. F. A., and Chou, J. T. Y., 1957. Quart. J. micr. Sci., **98**, 341.
Stoeckenius, W., 1957. Exp. Cell Res. **13**, 410.
Schmidt, W. T., 1939. Nova Acta Leopoldina, **7**, 1.
Young, J. Z., 1932. Quart. J. micr. Sci., **75**, 1.
—— 1953. Ibid., **94**, 399.
—— 1956. Endeavour, **15**, 5.

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